EFFECTS OF TEMPERATURE ON THE GROWTH AND PHYSIOLOGY OF EUCALYPTS

This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy of the Australian National University.

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Except where specific reference is made to the work of another person, the research reported in this thesis is original and was done without collaboration.

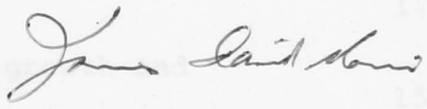

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TABLE OF CONTENTS

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ABSTRACT

Field trials have shown that eucalypts native to cool temperate areas of Australia may die or become distorted after several years of healthy growth when planted in warm temperate regions. The cause of failure of these species appears to be associated with the difference in temperature regime from that of their natural habitat, and a comparison of the growth of *E. regnans* and *E. grandis* seedlings at a range of temperatures in controlled environments demonstrated a similar effect on a shorter time scale, attributable to excessive growing temperatures. After 16 weeks of healthy growth at day/night temperatures of $30^{\circ}/25^{\circ}C$, the growth rate of *E. regnans* seedlings slowed to less than that attained at lower temperatures and symptoms of high temperature stress developed. Growth of *E. grandis* seedlings was fastest at $30^{\circ}/25^{\circ}C$, and even at $33^{\circ}/28^{\circ}C$, where some stress symptoms were evident, this species maintained vigorous growth while most *E. regnans* seedlings died within 16 weeks.

Measurements of net photosynthesis indicated a higher optimum temperature and less steep decline in CO_2 uptake at supra-optimal temperatures in *E. grandis*, but dark respiration rates were also higher in this species at temperatures above $24^{\circ}/19^{\circ}C$. Root respiration rates and respiratory quotients suggested that a root starvation effect may occur in both species at supra-optimal temperatures, possibly caused by retardation of transport processes.

Root saps extracted from the seedlings showed little difference between species in the effects of temperature on sugar, protein and amino acid concentrations. As there is evidence that plant growth substances could be involved in regulating the effects observed at supra-optimal temperatures, the concentrations of auxins, cytokinins, inhibitors and gibberellin-like promoters in the leaves and roots of both species were surveyed over a range of growing temperatures and seedling ages. Variation in all the substances assayed occurred with both age and temperature, but distinct differences between species which appeared to be correlated with the differing effects of temperature on growth and form were confined to inhibitors and promoters in the leaves.

More detailed fractionation and assay of leaf extracts revealed the presence of at least eight gibberellin-like promoters and six acid inhibitors in both species, most of which varied in concentration with both age and temperature. However, the overall promoting activity in *E. regnans* leaf extracts was found to fall by 50% with an increase in temperature from $24^{o}/19^{o}C$ to $30^{o}/25^{o}C$, while that of *E. grandis* extracts remains constant. At the same time, the overall inhibiting activity of *E. regnans* extracts also falls, but that of *E. grandis* extracts increases strongly.

The effects of temperature on growth substance concentrations are considered likely to be important in causing the effects on seedling growth and morphology observed, probably through changes in a complex balance of several growth regulators. The difference between species in optimum tempera-

ture and tolerance of supra-optimal temperatures may be primarily due to isoenzymatic differences occurring as adaptations to differing natural environments, and the decline in the apparent optimum temperature for growth of *E. regnans* may result from a combination of seasonal temperature effects with the exhaustion of food reserves.

CHAPTER 1

INTRODUCTION

1.1. Background

The increasing demand for short-fibred pulp by the Australian pulp and paper industry over the last two decades has resulted in the development of techniques for the intensive production of native hardwoods (Pryor *et al.* 1968). The increased use of direct seeding and especially planting of nursery-grown stock for establishment and regeneration of eucalypts has led to the possibility of establishing desirable species on sites outside the range of their natural occurrence: species can often be extended some distance beyond their natural range simply by removing competition from other eucalypts (Pryor 1976).

However, Pryor (1972) pointed out that the limits to the natural distribution of a species are the result of a number of environmental influences, some of which may continue to prevent successful establishment of the species on sites beyond its natural range, even in a cultivated, fertilised, weed-free, protected plantation situation. Without complete knowledge of the species' tolerance of environmental conditions beyond the range which apply in its natural habitat, there is no substitute for practical field trials in locations where establishment is proposed, to predict whether such a venture will be successful.

Such a trial was included by A. P. M. Forests Pty. Ltd. in an experimental eucalypt plantation programme in the

Coffs Harbour region of coastal northern New South Wales which commenced in 1958 (Pryor and Clarke 1964). The major species planted were *Eucalyptus grandis* Hill ex Maiden, *E. saligna* Sm. and *E. pilularis* Sm., all of which are native to the Coffs Harbour region, but small areas were planted with a wide variety of *Eucalyptus* species from different parts of Australia. The performances of 28 species over a 10 year period, summarised by Pryor (1972), reveal intriguing differences between species in ability to survive and grow in conditions somewhat removed from those prevailing in their natural habitats.

Of particular interest is the failure of some important timber species from the cool temperate forests of south-eastern Australia. These include *E. regnans* F. Muell. and *E. delegatensis* R.T. Bak., which died completely after 2 or 3 years growth, *E. nitens* Maiden which displayed grossly disordered and very poor growth, and *E. globulus* Labill. and *E. st. johnii* R.T. Bak. which also failed completely within a ten year period. Pryor (1972) cites examples of similarly disordered growth in overseas plantings of *E. globulus* and *E. maculata* Hook. A visit to the plantation site in November 1974 revealed the nature of this disorder in a few surviving trees of *E. st. johnii* and *E. sieberi* L. Johnson: the trees displayed excessive branch development, with many bends and kinks, leaf death in 60% or more of the crown, and copious outgrowth of epicormic shoots, many of which had also died. Even *E. saligna* from the most southern provenance planted showed the same symptoms in a milder form, and was not expected to survive for a further 10 years. A particularly

interesting aspect of the failure of these species was the initial period of healthy and vigorous growth prior to the development of the symptoms described: for example, *E. regnans* and *E. delegatensis* reached heights of up to 10 m in 2 to 3 years before they died.

1.2. Temperature and eucalypt growth

Pryor (1972) considered the disordered growth and eventual death of cool temperate species planted at Coffs Harbour to be associated in some way with the difference in temperature regime from that of the natural range of the species. Of the major environmental factors which commonly influence plant growth and development, temperature differences between the natural habitats and the Coffs Harbour site are certainly the most likely cause of the effects observed. There is evidence (Pryor 1972) that no precise day-length requirement exists for *Eucalyptus* spp., although Scurfield (1961) found that growth of 7 species was slowed by short (8 hour) photoperiods and Eldridge (1969) obtained similar results for *E. regnans*. Mean annual rainfall at Coffs Harbour is approximately 1650 mm, well within the range falling on natural sites of the Victorian and Tasmanian species tested; e.g. *E. regnans*, a species of particularly limited distribution, occurs naturally on sites where annual rainfall is between 1000 and 2000 mm. Similarly the distribution of rainfall through the year is unlikely to limit growth of the southern species, most of which occur in regions of fairly uniform monthly rainfall: although the Coffs Harbour region has a summer rainfall maximum, average rainfall in the driest month (September) exceeds 70 mm (Anon. 1966). The satisfact-

ory early growth of southern species on the trial site, and the continuing rapid growth of those native to the area, suggest that neither soil conditions nor competition placed a limit on the growth of the planted eucalypts: if site preparation and fertilising procedures such as those described by Pryor *et al*. (1968) were applied, the potential limitations due to podsolic soils of low nutrient status and the presence of a heavy grass sward would certainly be avoided.

Assuming that the temperature regime of the trial site was responsible for the difference in growth and form of the southern eucalypts from those normally displayed on sites in the natural range, there are several aspects of this regime which could individually or together bring about the effects observed; these include day temperature, night temperature and both diurnal and seasonal temperature variations. Of these, only diurnal variation appears unlikely to be of importance: using the difference between monthly mean maximum and minimum temperatures as an index, the day-night differential at Coffs Harbour varies from 8^{o}C in January to 12^{o}C in July (Commonwealth Bureau of Meteorology records). Again using *E. regnans* as a representative species of those which failed at Coffs Harbour, a natural site studied by Cremer (1975) had corresponding summer and winter differentials of 14^{o}C and 6^{o}C respectively, while data listed by Brown and Hall (1968) for several Victorian and Tasmanian localities within the natural range of this species reveal differentials between 9 and 14^{o}C in January and between 7 and 10^{o}C in July. The diurnal variation in temperature at Coffs Harbour thus does not differ greatly from that which occurs in the natural habitat, and results obtained by Cremer (1975), Blake (1973) and Scurfield (1961)

suggest that eucalypts do not possess a high degree of thermoperiodic sensitivity.

Pryor (1972) likened the apparently temperature-induced failure of southern species at Coffs Harbour to another effect displayed in the same trial, namely that species can be transferred from summer rainfall to winter rainfall areas but not in the reverse direction. *E. saligna*, *E. citriodora* Hook. and other species from northern New South Wales and Queensland have indeed been grown satisfactorily in the southern states (as ornamentals or specimen trees). This is not the paradox it may seem: if excessively high temperatures are assumed to cause the distorted growth and early death of cool temperate species planted in warm temperate regions, a steeper decrease in growth rate at temperatures above the optimum than below it combined with a simple difference in optimum temperature between northern and southern species could possibly account for the successful transfer of species in one direction but not the other.

Whether such a species difference in response to temperature changes in fact exists has not yet been demonstrated, as only a few studies have been directed toward exploring the effects of temperature on the growth and development of eucalypts. Scurfield (1961) carried out the first such study, comparing the growth and morphology of 7 species (all from south-eastern Australia) under various day/night temperature regimes and daylengths. Both day and night temperatures were shown to influence growth and form, and best growth was found at about 25-27^{o}C day temperature with night temperatures

3°C or 5°C lower. The results of this study lacked precision as a result of the limited facilities available for growth of plants in controlled environments at the time.

With the opening of the CERES phytotron in Canberra (Morse and Evans 1962), excellent controlled environment facilities became available and these have been utilised in subsequent studies of the effects of temperature on the growth and development of eucalypts. Green (1969) studied temperature responses of *E. pauciflora* Sieb. ex Spreng. from different altitudinal populations; he raised seedlings at five day temperatures over the range 15 to 33°C, with night temperatures 5°C lower, and found optimal height growth at 24°C 19 weeks after sowing. Growth at 30°C was slightly better than at 24°C at first but fell behind after about 16 weeks, while 33°C was clearly supra-optimal, seedling height falling behind that at 18°C after 12 weeks and 15°C by 17 weeks. No significant difference in optimum or minimum temperatures for growth was found between altitudinal populations.

Eldridge (1969) included a similar comparison of growth at 8 different day temperatures over the range 9 to 36°C, again with a 5°C day-night differential, in his study of altitudinal variation in *E. regnans*. He found a difference in temperature optima between families from different altitudes, and also a decrease in the optimum day temperature with time, from above 30°C at 8 to 9 weeks post-germination to around 24°C 4 weeks later. This resembles the results obtained by Green (1969) for *E. pauciflora*.

Blake (1973), seeking an effect of thermoperiod (i.e. day-night temperature differential) on growth and form of *E. obliqua* L'Herit., grew seedlings at seven temperature regimes with day temperatures of 10°C, 24°C or 28°C and night temperatures between 0° and 23°C lower. He found differences in height growth and form between regimes with different temperature differentials, maximum height growth occurring at 28°/18°C, 24°/24°C and 24°/18°C. Experiments of this kind cannot usually distinguish between the effects of night temperature and those of day-night differential, especially since interactions between the differential and the day and/or night temperatures are likely to exist. However, it is clear that no strong thermoperiodic requirement as demonstrated by Kramer (1957) in *Pinus taeda* exists in *E. obliqua* seedlings.

Cremer (1975) studied the effects of temperature and other factors on shoot development and growth of *E. regnans* seedlings in the field and in controlled environments. His phytotron study extended the work of Eldridge (1969), and produced similar results : after 7 weeks at temperature regimes ranging from 9°/4°C to 33°/28°C, maximum height and dry weight was attained by seedlings grown at 24°/19°C. The response of *E. regnans* to day-night temperature differentials was also examined by comparing growth at night temperatures of 10°C, 16°C, 22°C and 31°C, with a day temperature of 21°C in each case. Results indicated that *E. regnans*, like *E. obliqua*, does not display a high degree of thermoperiodic sensitivity.

The effects of the 21°/31°C regime on growth and form resembled those found at 33°/28°C, including in particular

excessive growth of branches; Blake (1973) observed the same effect in *E. obliqua* at 28°/28°C, and it was also characteristic of the trees which failed in the Coffs Harbour species trial. High night temperatures (28°C and above) thus appear to be responsible for this effect at least, although temperature regimes such as 33°/22°C, which combine a high day temperature with a night temperature known to allow normal growth, have not been tested and may cause the same effect. At Coffs Harbour, with a mean minimum temperature of about 19°C in the warmest month (Commonwealth Bureau of Meteorology records), night temperatures would seldom be expected to exceed 28°C.

Studies of the effects of temperature on physiological characteristics of eucalypts other than growth and form are few in number. Hofstra and Hesketh (1969) included *E. regnans* with a number of agricultural crop species in a study of the effect of temperature on stomatal aperture, demonstrating a steady increase in aperture with temperature over the range 15°to 36°C. Slatyer and Ferrar (1977) recently described a study of the variation in photosynthesis of altitudinal populations of *E. pauciflora* with growing temperatures in the range 8°/4°C to 33°/28°C. They found optimum temperatures for three populations between 22°C and 26°C, increasing as the altitude of the parent population decreased. Slatyer (1977) continued this study and examined the effect of preconditioning seedlings at high or low temperatures on the optimum temperature for photosynthesis: the optimum was found to shift in each case towards the preconditioning temperature.

[1] The two species chosen differ considerably in a number of morphological features, and are considered to belong to different subgenera (Pryor and Johnson 1971). This difference is reflected in the slower germination and growth of *E. regnans* observed in the growth study described in chapter 2, and possibly in other species differences found. Other southern species which are taxonomically closer to *E. grandis* (*e.g. E. st. johnii*) might be expected to display less difference in growth characteristics up to their optimum temperature; however, the results of the Coffs Harbour species trial indicate that the effects on southern species ascribed here to supra-optimal temperature are independent of taxonomic group.

1.3. Approach to the study

On the basis of the evidence discussed above, differences in temperature preference were assumed to be a major cause of the difference between southern and northern eucalypts in ability to survive on the Coffs Harbour trial site. The first objective of this project was therefore to compare the growth response of representative northern and southern species to a range of temperatures, seeking in particular a difference in optimum temperature and in the decline in growth rate at higher and lower temperatures. As it has been shown that the optimum temperature for growth of some species decreases with increasing seedling age, this effect should be taken into account in making species comparisons, and growth monitored for a long enough period to ensure that the temperature responses found give a valid indication of the optimum temperature for each species.

Eucalyptus regnans and *E. grandis* were chosen as representative species of the cool temperate and warm temperate climates respectively.[1] *E. regnans* occurs naturally in southern Victoria and Tasmania, best growth occurring in mountain valleys where rainfall exceeds 1100 mm p.a.; *E. grandis* is native to the east coast from central New South Wales to south-eastern Queensland, with localised occurrences further north, and is often found on alluvial river flats subject to flooding. The climate in this range is warm temperate to sub-tropical, with annual rainfall of 1000-1700 mm (Brown and Hall 1968). Seed of *E. regnans* was collected near Dover, Tasmania, and that of *E. grandis* collected at Coffs Harbour, at an altitude of approximately 100 m in each case.

After information on the growth response of each species to temperature had been obtained, the second objective of this project was to examine the effects of temperature on aspects of the physiology of the two species. In this way it might be possible to locate the physiological basis of the difference in temperature preferences between southern and northern species, which could in turn provide valuable information on mechanisms of heat tolerance in eucalypts and other species. The involvement of differences in seasonal temperature variation between cool temperate and warm temperate regions, as mentioned earlier, was not examined, but the possible role of such differences in causing the observed growth effects will be discussed in Chapter 11.

CHAPTER 2

TEMPERATURE EFFECTS ON THE GROWTH AND DEVELOPMENT OF *EUCALYPTUS REGNANS* AND *E. GRANDIS* SEEDLINGS

2.1. Introduction

An investigation of the apparent difference in the maximum temperatures for growth of *E. regnans* and *E. grandis* as suggested by the Coffs Harbour species trials (Pryor 1972) requires firstly a comparative study of the growth of the two species at a range of temperatures. This range needs to be broad enough to encompass the temperature optimal for growth of each species, and provide an indication of the effects on growth and development of temperatures above and below the optima. Such a study will reveal whether these representative "northern" and "southern" eucalypts differ in their optimum or maximum temperatures, or both.

It is also desirable that the growth study should be continued for a realistically long period, as the temperature range and optimum for a species may change with the transition from seedling to mature plant. Such a change in the optimum temperature for growth of *E.regnans* has already been demonstrated by Eldridge (1969). The involvement of an age effect in the failure of *E. regnans* and other species at Coffs Harbour is suggested by the period of healthy growth which preceded the development of stress symptoms leading to death of the trees.

A study of the effects of temperature on growth could be carried out either by establishing field trials over a wide range of locations, or by the use of controlled environments in a phytotron. The former approach offers the advantage of "real world" conditions, so that the results of the trial could be directly applied in planning for the "off-site" establishment of the species involved. There are however a number of disadvantages, of which two are particularly important: first, the wide separation of locations necessary to cover an adequate temperature range while keeping other major factors such as annual rainfall and soil type broadly constant would severely limit the frequency of observation of the trials. Secondly, it is extremely difficult to eliminate the effects of all environmental factors other than temperature in such an experiment, so confounding of the effects is certain to occur, probably to such an extent that no firm conclusions as to the effects of temperature on growth can be drawn.

A controlled environment approach readily overcomes these difficulties, offering control of growing temperature in almost complete isolation from other factors, essential if differences in growth are to be definitely attributed to the effects of temperature alone. As the conditions used in such an experiment (e.g. regular watering and nutrient supply, freedom from predators and competing vegetation) are likely to be more favourable than experienced in the field at any location, the results can only be applied in establishment planning to a limited extent and with caution: the optimum temperature for growth of a species in the phytotron may not

be optimal at a proposed planting site in the field. The use of controlled environments has the further disadvantage that, unless large growth rooms are available, the time span of the growth study is limited to the time taken by the plants to reach the maximum manageable size in the glasshouses or growth chambers used. The rapid growth rates attainable under phytotron conditions, particularly at near-optimal temperatures, may allow the plants to reach this size in a few months; on the other hand, fast growth rates may enable the plants to reach a given stage of development (e.g. the onset of stress symptoms in *E. regnans* due to supra-optimal temperatures) much sooner than they would under field conditions, thereby shortening the time span necessary for the growth study.

Among the factors held constant in a phytotron-based growth study are two which relate to growing temperature and hence deserve special consideration. These are the day-night temperature differential, and the thermoperiod. In a field-based study, the use of natural temperatures implies that these are allowed to vary freely, even if all other factors can be held constant between sites. However, the results of Cremer (1975), Blake (1973) and Scurfield (1961) discussed in Chapter 1 suggest that eucalypts do not possess a high degree of thermoperiodic sensitivity and in any case the mean day-night differential at Coffs Harbour is within the range occurring on natural sites of *E. regnans* and other species in the southern states. For the purposes of this study, both thermoperiod and day-night differential were therefore held constant, and the growth effects observed are those due to temperature alone.

2.2. Materials and methods

2.2.1. Plant materials and cultural conditions

All seedling material for the growth study and subsequent experiments was raised in the C.S.I.R.O. Canberra phytotron (Morse and Evans 1962). Seeds of *E. regnans* and *E. grandis* were sown in 1:1 perlite/vermiculite in shallow trays and placed in a naturally-lit, air-conditioned glasshouse maintained at a day/night temperature regime of $24^{\circ}/19^{\circ}C$. Photoperiod in the phytotron glasshouses was 14 hours, natural daylength being extended as necessary by the use of supplementary incandescent lighting, but the thermoperiod was 8 hours day, 16 hours night.

E. grandis seedlings emerged after 8 days, but *E. regnans* was slower, the first seedlings appearing after 13 days. When the seedlings had one pair of true leaves open and were beginning to develop a second pair they were transplanted into pots of perlite/vermiculite (two or three seedlings per pot) and returned to the $24^{\circ}/19^{\circ}C$ glasshouse. In *E. grandis* this stage was reached 2½ weeks after sowing, while the slower-growing *E. regnans* seedlings were not potted until 6 weeks after sowing. The seedlings were allowed to become established in the pots over a period of 2 weeks at $24^{\circ}/19^{\circ}C$, then equal numbers of pots were transferred to glasshouses at day/night temperatures of $18^{\circ}/13^{\circ}C$, $21^{\circ}/16^{\circ}C$, $24^{\circ}/19^{\circ}C$, $30^{\circ}/25^{\circ}C$ and $33^{\circ}/28^{\circ}C$. The seedlings were thinned to one per pot when they became large enough to interfere with each other's growth, leaving an essentially uniform population of seedlings for observation and harvest. Thinning took place two weeks after

[1] Mortality of *E. regnans* at 33° / 28°C reduced the number of seedlings available for measurement to 8 by 14 weeks after transfer. The exclusion of dead seedlings from the sample introduces a positive bias in the results for *E. regnans* at this temperature, but this does not affect the conclusions to be drawn as growth of this species at 33° / 28°C was well below that observed at lower temperatures in any case.

transfer of *E. grandis* and four weeks after transfer of *E. regnans*.

All pots were irrigated once daily with a complete nutrient solution, and additional watering was given once daily at day temperatures below 30°C and twice daily at the higher temperatures. Seedlings were repotted into larger pots as they outgrew the 12 cm pots used initially, up to 23 cm pots towards the end of the study. Height growth of both species was rapid, especially at the higher temperatures, but stem thickening lagged behind so that the seedlings were weak and staking was necessary soon after transfer.

Seedlings were harvested from the 24°/19°C glasshouse within a week of the transfer of seedlings to other temperatures, and from all glasshouses at regular intervals thereafter. *E. regnans* seedlings were harvested at 8, 12, 16 and 20 weeks after transfer, but the very rapid growth of *E. grandis* at 30°/25°C and 33°/28°C made it impossible to maintain seedlings of this species in a glasshouse longer than 16 weeks; a ten week harvest therefore replaced the twenty week harvest at these two temperatures.

2.2.2. Assessment of growth and development

Fortnightly observations of the seedlings were made from the time of transfer throughout the growth study. The heights of 12 randomly selected plants of each species were recorded[1], and the condition of the plants in terms of leaf size, colour, necrosis and abscission, branch size, angle and dieback, stem thickness, development of epicormic shoots and general health was noted.

At each harvest, 5 seedlings were selected at random from each temperature regime and the height, number of stem leaf pairs differentiated and number of primary branches on each were recorded. The water potential of the youngest fully expanded leaf of each seedling was estimated by measuring the pressure required to cause sap to exude from the cut petiole in a small hydraulic press (Campbell Scientific, Inc. model J-14). The stem leaves of each seedling were then removed and weighed, and their area determined by means of an automatic leaf area meter. This was repeated with the branch leaves, except that in the measurement of larger seedlings, which frequently possessed 1500 leaves or more, leaf area of a sample of 100 leaves was measured and the mean leaf area multiplied by the total number of leaves present. Test measurements indicated that this sample size gave an accurate estimate of total leaf area.

The branches were next removed and weighed, followed by the stem. Perlite and vermiculite were shaken and washed from the root system, which was blotted dry and weighed also. The leaves, branches and chopped root systems of all 5 seedlings were bulked, and five samples of 1 to 2 g of each, plus segments of the 5 stems, were oven-dried to constant weight at 105^{o}C for estimation of the moisture content of each part. Dry weight distribution by parts was calculated from the fresh weight and moisture content data.

Also at each harvest, leaf buds and root tips were collected from the plants for measurement of respiration rates and respiratory quotients (Chapter 3), and the bulked leaves

and roots were stored at -20°C for later extraction and analysis (Chapters 6 and 7). An additional 4 or 5 seedlings were removed from the glasshouses at each harvest for extraction of root saps (Chapter 4).

2.3. Results

2.3.1. Height growth and qualitative observations

The fortnightly measurements of height of the seedlings are recorded in Figures 2-1 and 2-2. At the beginning of the experiment height growth of *E. grandis* increased with temperature up to 33°/28°C, but after about 4 weeks 30°/25°C became established as the optimal temperature regime of those tested. By the sixteenth week the seedlings at 30°/25°C were considerably taller than those at 33°/28°C, with the other temperature regimes further behind. While seedlings could not be kept in the glasshouses at the higher temperatures beyond this time, it is apparent from Figure 2-1 that 30°/25°C would have remained optimal for considerably longer, perhaps indefinitely.

For *E. regnans*, 33°/28°C was inferior to 30°/25°C from the first measurement, and after 4 weeks height growth at the higher temperature was even less than at 18°/13°C. The development of *E. regnans* at 33°/28°C was also characterised by frequent seedling mortality, absent from all other temperatures, so that all surviving seedlings were harvested by 16 weeks after transfer. Height growth at 24°/19°C and 30°/25°C were approximately equal up until the sixteenth week, after

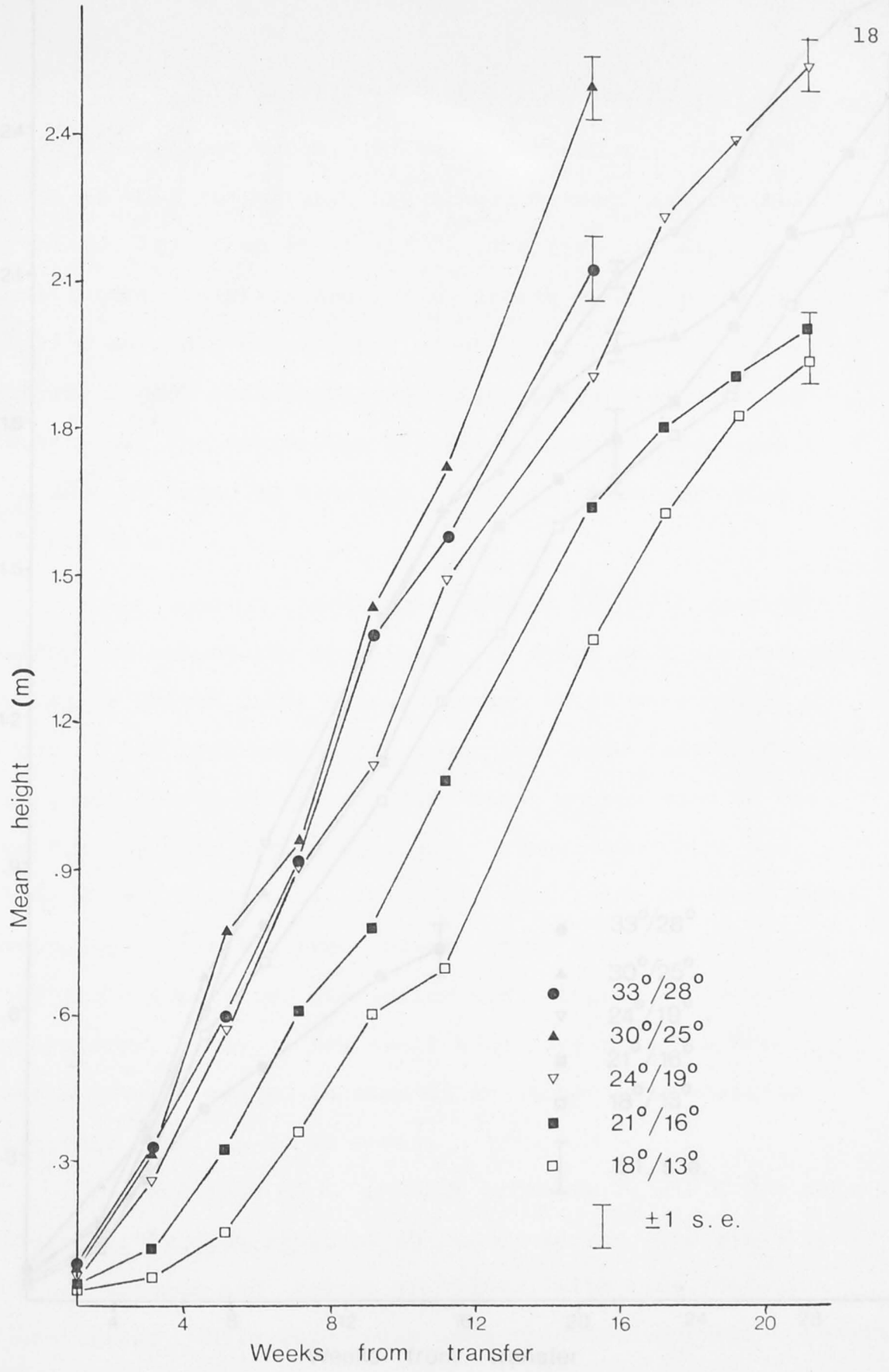


Figure 2-1. Height growth of *E. grandis* seedlings over a period of 20 weeks at different temperature regimes. Each point is the mean of 12 seedlings.

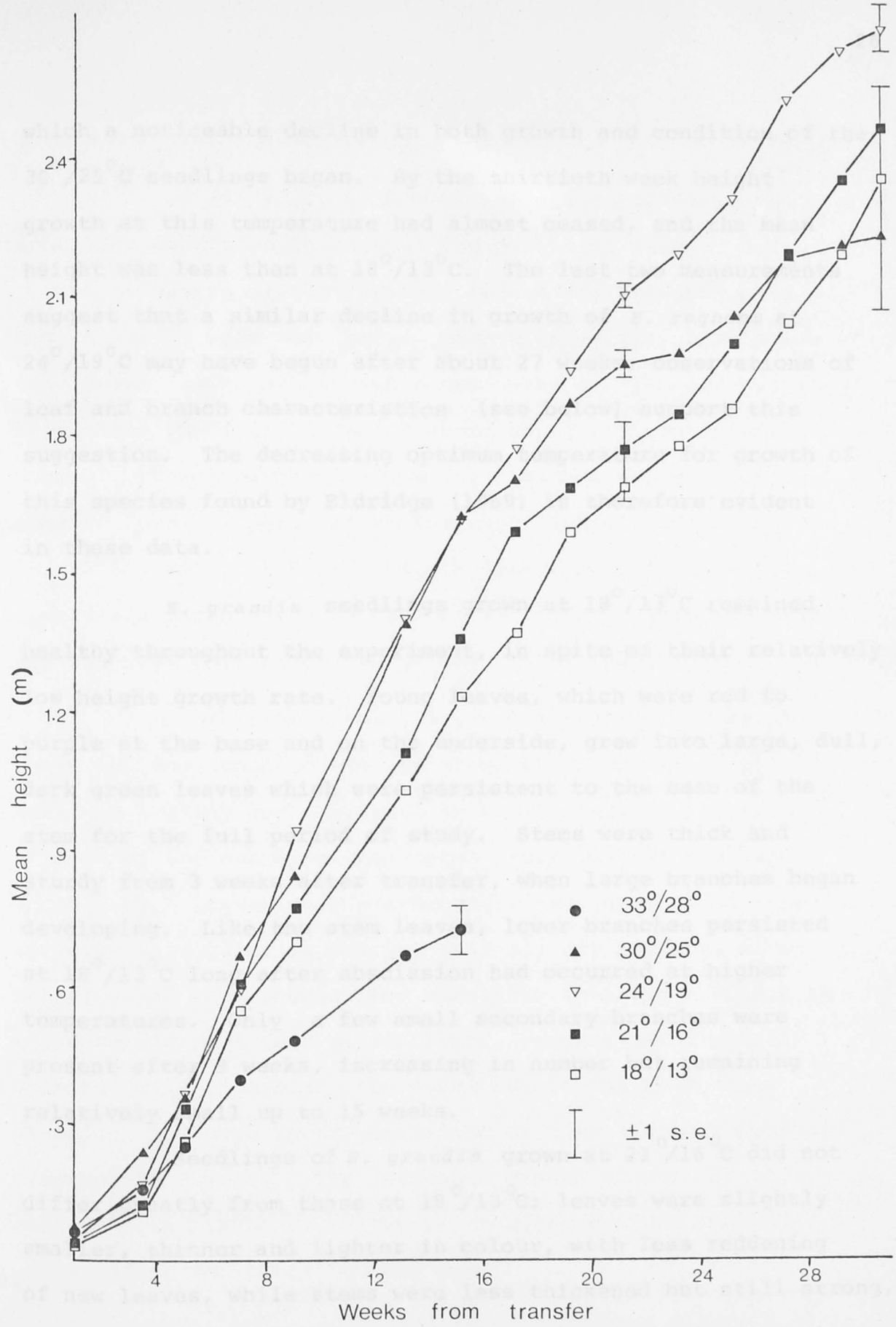


Figure 2-2. Height growth of *E. regnans* seedlings over a period of 30 weeks at different temperature regimes. Each point is the mean of 12 seedlings up to 21 weeks from transfer, and 4 seedlings thereafter.

which a noticeable decline in both growth and condition of the $30^{\circ}/25^{\circ}$C seedlings began. By the thirtieth week height growth at this temperature had almost ceased, and the mean height was less than at $18^{\circ}/13^{\circ}$C. The last two measurements suggest that a similar decline in growth of *E. regnans* at $24^{\circ}/19^{\circ}$C may have begun after about 27 weeks; observations of leaf and branch characteristics (see below) support this suggestion. The decreasing optimum temperature for growth of this species found by Eldridge (1969) is therefore evident in these data.

E. grandis seedlings grown at $18^{\circ}/13^{\circ}$C remained healthy throughout the experiment, in spite of their relatively low height growth rate. Young leaves, which were red to purple at the base and on the underside, grew into large, dull, dark green leaves which were persistent to the base of the stem for the full period of study. Stems were thick and sturdy from 3 weeks after transfer, when large branches began developing. Like the stem leaves, lower branches persisted at $18^{\circ}/13^{\circ}$C long after abscission had occurred at higher temperatures. Only a few small secondary branches were present after 9 weeks, increasing in number but remaining relatively small up to 15 weeks.

Seedlings of *E. grandis* grown at $21^{\circ}/16^{\circ}$C did not differ greatly from those at $18^{\circ}/13^{\circ}$C; leaves were slightly smaller, thinner and lighter in colour, with less reddening of new leaves, while stems were less thickened but still strong, with large drooping primary branches. Leaves on the lowest branches died and were dropped, followed by abscission of the branches themselves, from about 9 weeks on. Secondary branch

development was slower at this temperature than at $18^{o}/13^{o}C$.

At $24^{o}/19^{o}C$, *E. grandis* seedlings remained in excellent condition throughout the period of study. Only slight reddening of the youngest leaves was apparent, and mature leaves were very large, smooth and soft. Abscission of the lowest leaves began within two weeks of transfer, followed by abscission of the lower branches so that crowns remained compact. Development of secondary branches was more rapid than at lower temperatures. The condition of seedlings at $30^{o}/25^{o}C$ was much the same, their larger size making them appear even more handsome than at $24^{o}/19^{o}C$. Secondary branching was also more developed at the higher temperature.

E. grandis seedlings grown at $33^{o}/28^{o}C$ were above their optimum temperature, as shown by Figure 2-1, but remained healthy and continued to grow vigorously in spite of effects of the supra-optimal temperature on leaf and branch morphology. Leaves were considerably smaller than at other temperatures, paler in colour, dull and dry looking, often wrinkled and sometimes showed chlorotic mottling. Branching was more vigorous than at the lower temperatures, primary branches with elongated internodes developing soon after transfer, followed by the formation of many strong secondary branches by 9 weeks. The increased branch growth led to a much greater number of leaves at this temperature regime, compensating for their reduced size.

E. regnans seedlings at $18^{o}/13^{o}C$ and $21^{o}/16^{o}C$ remained healthy and vigorous for the whole period of study. Leaves were large, soft, bright green and glossy; at $18^{o}/13^{o}C$

most of the stem leaves and lower branches were still alive after 30 weeks, but at 21°/16°C abscission of lower leaves and branches had begun by 19 weeks from transfer. Branch development was more rapid at 21°/16°C, a few small secondary branches forming by the seventh week, while at 18°/13°C they were just beginning to grow after 19 weeks. By the twenty-seventh week tertiary branches were present at 21°/16°C and just appearing at 18°/13°C. At both temperature regimes a few small epicormic branches developed in the upper crowns from the twentieth week on.

Growth of *E. regnans* at 24°/19°C was also vigorous throughout the study, the seedlings resembling those grown at 21°/16°C in leaf characteristics, with slightly less sturdy stems and slower development of branches; primary branches were initially not as strong as at lower temperatures, and secondary branching was not well developed until 27 weeks after transfer. Abscission of lower leaves and branches was only slight up to the twentieth week, but by 25 weeks some lower branches were dying back and epicormic shoots arising in their place. One tree developed long straggly lower branches at 21 weeks, and by 27 weeks the branches on all trees were larger and more drooping than at lower temperatures. By 30 weeks some leaf death in the middle crowns had begun occurring; these leaf and branch effects resemble more marked effects shown by *E. regnans* at 30°/25°C and 33°/28°C (see below) and may have been the first symptoms of a general degrade leading to death of the seedlings.

Symptoms of high temperature stress were displayed by *E. regnans* at 33°/28°C soon after transfer from 24°/19°C. After 3 weeks widespread death of the lower leaves was occurring, sometimes extending further up the seedling, and all the plants were very limp. By 7 weeks several seedlings had died, and the others looked stunted and unhealthy. Leaves were small, narrow and dull light green in colour, while stem and branch growth appeared disordered, with frequent bends and kinks. After 13 weeks the remaining seedlings were obviously dying, with epicormic shoots replacing dead leaves at points of abscission. Branches were longer and stronger than the stems, which offered virtually no support to the plants. Secondary branch development was more pronounced than at lower temperatures. The last surviving seedlings were harvested at 16 weeks, but it is unlikely that *E. regnans* could have survived much longer at this temperature.

The development of *E. regnans* seedlings at 30°/25°C was particularly interesting, in that a period of healthy growth preceded the appearance of stress symptoms similar to those seen at 33°/28°C. These seedlings remained healthy and vigorous throughout the first 16 weeks of the study. They were overall quite similar to the 24°/19°C plants during this period, except for having longer branches. By the nineteenth week however a change had taken place: new leaves were smaller, soft and dry to touch, and leaf shape also appeared to change with height of the seedling, becoming more narrowly lanceolate towards the top. Wrinkling and death of whole leaves occurred, beginning from the leaf tips or intervenal patches. This was

most pronounced in the lower crown, where it led to dieback of the branches and formation of stem and branch epicormic shoots. Branch growth was very vigorous, with many strong secondary branches and well developed tertiary branch buds. The older branches of the lower crown were however not excessively large. Stems were narrow and very lax, so much so that it was not immediately possible to distinguish the leading shoot from the upper branches.

The condition of the seedlings at $30^{o}/25^{o}C$ continued to deteriorate for the remaining 11 weeks of the study period. Leaf death occurred throughout the crown, followed by death of the lower branches and copious epicormic branching. Upper branch growth was excessive, with decreased branching angle and very weak stems. Tertiary branches were well developed by 27 weeks after transfer, and quaternary branching had begun. By 29 weeks bark symptoms were apparent: brown patches formed, enlarged then merged together. This led to death of the bark, followed by splitting and eventual decortication. No mortality had occurred by the end of the experiment (30 weeks after transfer) but it was clear that seedling death was inevitable within a few weeks or months.

2.3.2. Other growth effects

Variations in total dry weight and leaf area of the seedlings with temperature at the 16 week harvest are shown in Figures 2-3 and 2-4. Both graphs are of the same form, which also closely resembles a plot of height versus temperature at the same age: dry weight and leaf area of *E. grandis* seedlings increase with rising temperature to a pronounced

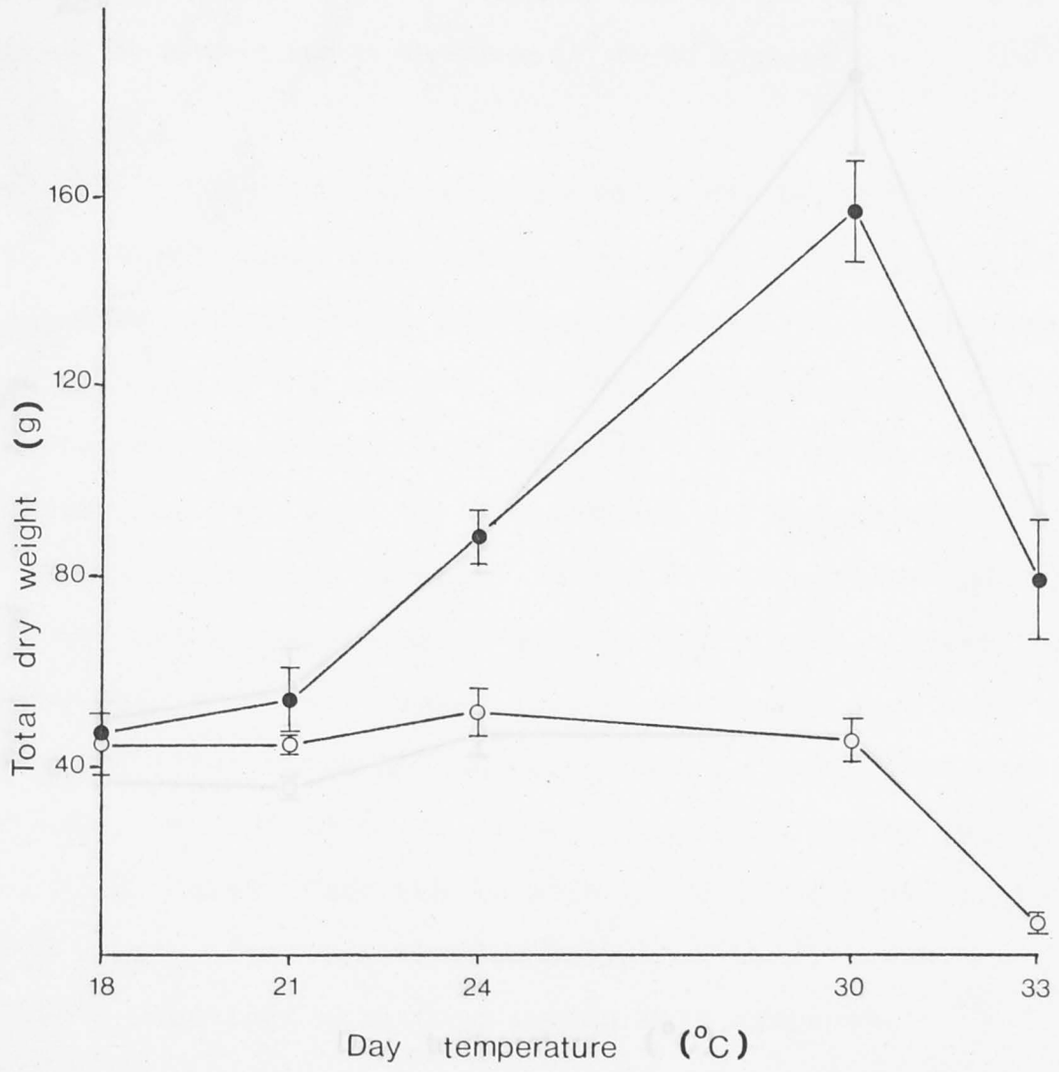


Figure 2-3. Total dry weight of seedlings of *E. regnans* (o) and *E. grandis* (●) after 16 weeks at different temperatures. Each point is the mean of 5 seedlings, ± standard error.

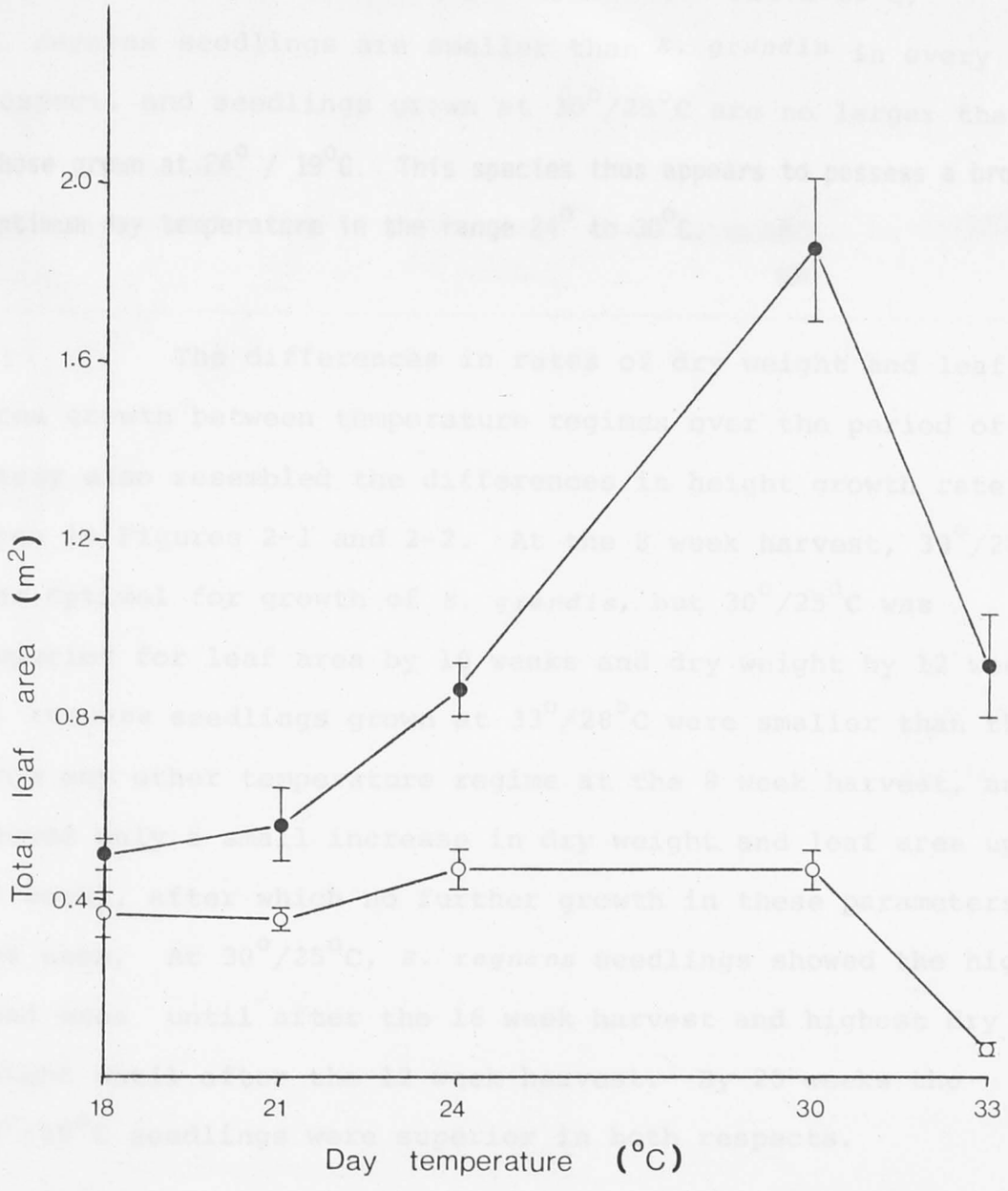


Figure 2-4. Total leaf area of seedlings of *E. regnans* (o) and *E. grandis* (●) after 16 weeks at different temperatures. Each point is the mean of 5 seedlings, ± standard error.

optimum at 30°/25°C. At day temperatures above 18°C, *E. regnans* seedlings are smaller than *E. grandis* in every respect, and seedlings grown at 30°/25°C are no larger than those grown at 24° / 19°C. This species thus appears to possess a broad optimum day temperature in the range 24° to 30°C.

The differences in rates of dry weight and leaf area growth between temperature regimes over the period of study also resembled the differences in height growth rate seen in Figures 2-1 and 2-2. At the 8 week harvest, 33°/28°C was optimal for growth of *E. grandis*, but 30°/25°C was superior for leaf area by 10 weeks and dry weight by 12 weeks. *E. regnans* seedlings grown at 33°/28°C were smaller than those from any other temperature regime at the 8 week harvest, and showed only a small increase in dry weight and leaf area up to 12 weeks, after which no further growth in these parameters was seen. At 30°/25°C, *E. regnans* seedlings showed the highest leaf area until after the 16 week harvest and highest dry weight until after the 12 week harvest. By 20 weeks the 24°/19°C seedlings were superior in both respects.

The effects of temperature on dry weight and leaf area growth can be examined in more detail by the methods of growth analysis originally proposed by West *et al.* (1920). The mean relative growth rate (RGR) of each species between the 8 and 12 week harvests at each temperature is plotted in Figure 2-5. These data represent seedling growth at a fairly early stage of the experiment, but as the growth analysis relationships strictly only hold during the exponential growth

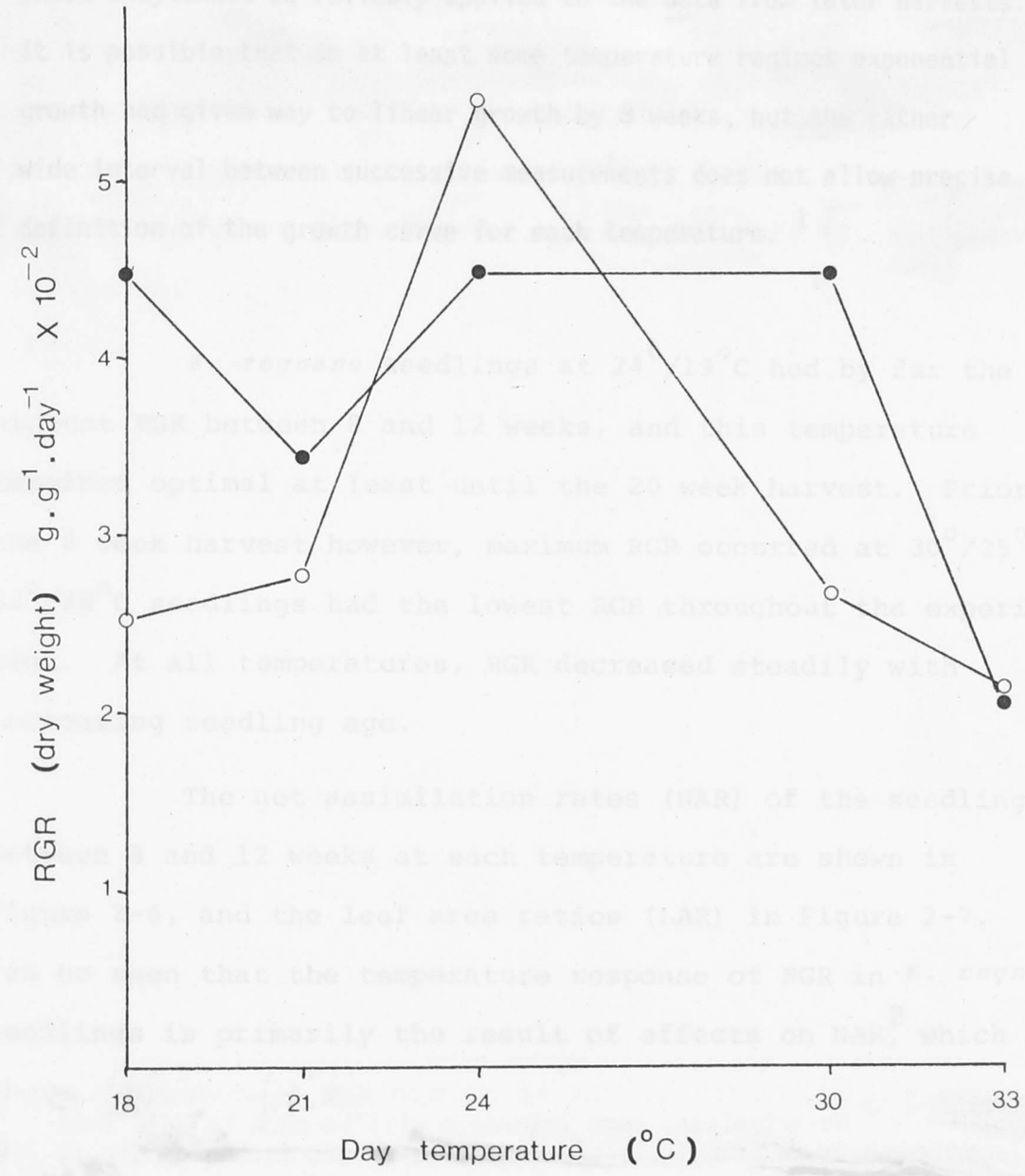


Figure 2-5. Relative dry weight growth rate of seedlings of *E. grandis* (●) and *E. regnans* (○) after 10 weeks at different temperatures. Based on mean dry weight data from 5 seedlings.

[1] Nevertheless, some reference will be made to data from later harvests to illustrate apparent time trends in the growth analysis parameters.

[2] Growth analysis techniques enable the effects of temperature on RGR to be classified as "caused by effects on NAR" or "caused by effects on LAR". While such a classification provides a useful basis for comparisons between species, it should not be taken to imply direct causality in a physiological sense. As the growth analysis parameters are derived wholly from measurements of dry weight and leaf area, they cannot be expected to identify the mechanisms underlying changes in growth rate.

phase they cannot be reliably applied to the data from later harvests. It is possible that in at least some temperature regimes exponential growth had given way to linear growth by 8 weeks, but the rather wide interval between successive measurements does not allow precise definition of the growth curve for each temperature. [1]

E. regnans seedlings at 24°/19°C had by far the highest RGR between 8 and 12 weeks, and this temperature remained optimal at least until the 20 week harvest. Prior to the 8 week harvest however, maximum RGR occurred at 30°/25°C; 33°/28°C seedlings had the lowest RGR throughout the experiment. At all temperatures, RGR decreased steadily with increasing seedling age.

The net assimilation rates (NAR) of the seedlings between 8 and 12 weeks at each temperature are shown in Figure 2-6, and the leaf area ratios (LAR) in Figure 2-7. It can be seen that the temperature response of RGR in *E. regnans* seedlings is primarily the result of effects on NAR, [2] which also shows a pronounced maximum at 24°/19°C. The slightly greater LAR at 30°/25°C (compared with 24°/19°C) is insufficient to counteract the low NAR, so RGR declines above 24°/19°C. The low LAR at 18°/13°C causes RGR to be less than at 21°/16°C, even though there is little difference in NAR between these temperature regimes. NAR decreased with increasing seedling age, as did LAR after the 12 week harvest; the declining RGR is due mainly to the relatively rapid decrease in NAR with time.

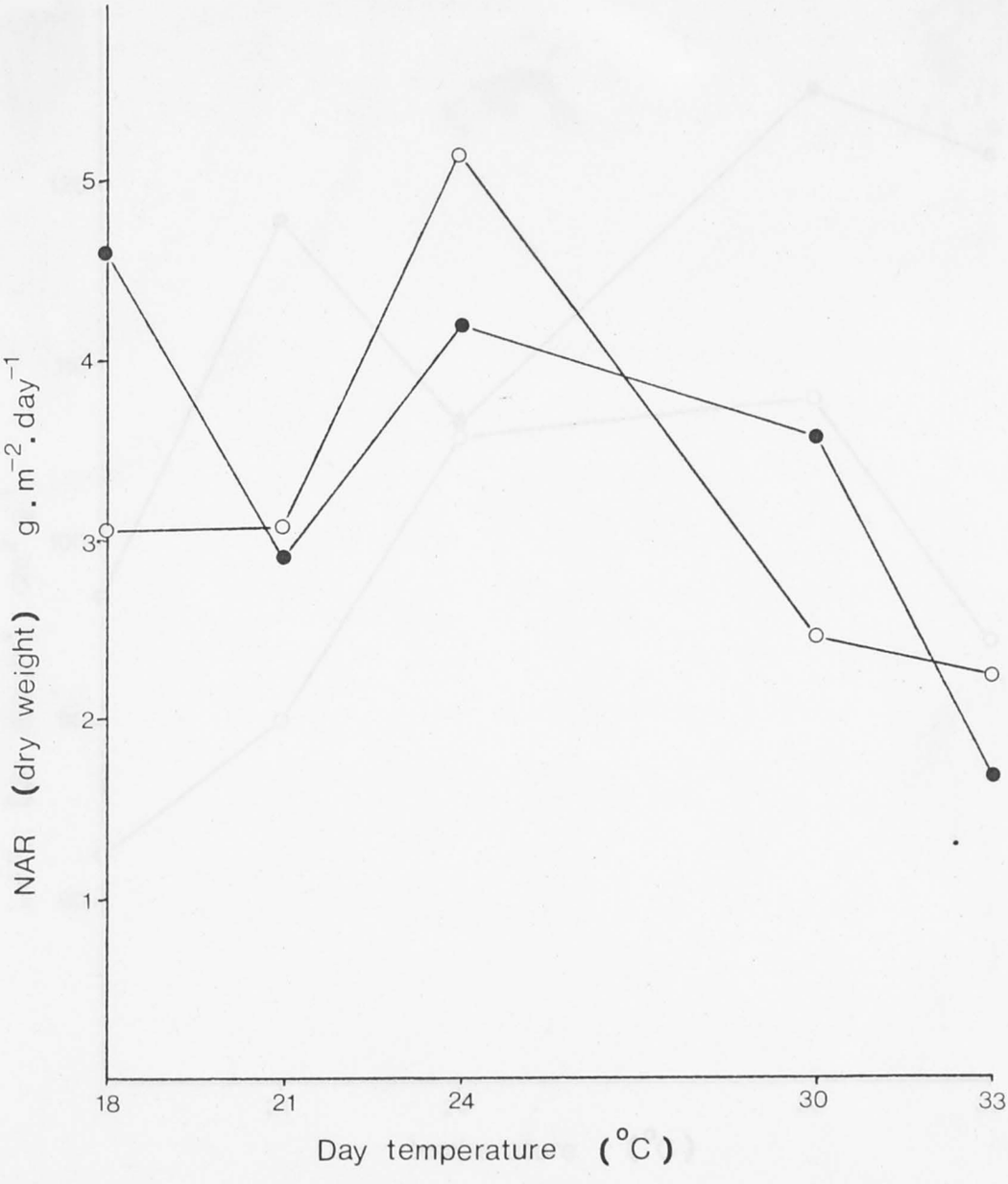


Figure 2-6. Net assimilation rate of seedlings of *E. regnans* (○) and *E. grandis* (●) after 10 weeks at different temperatures. Based on mean leaf area and dry weight data from 5 seedlings.

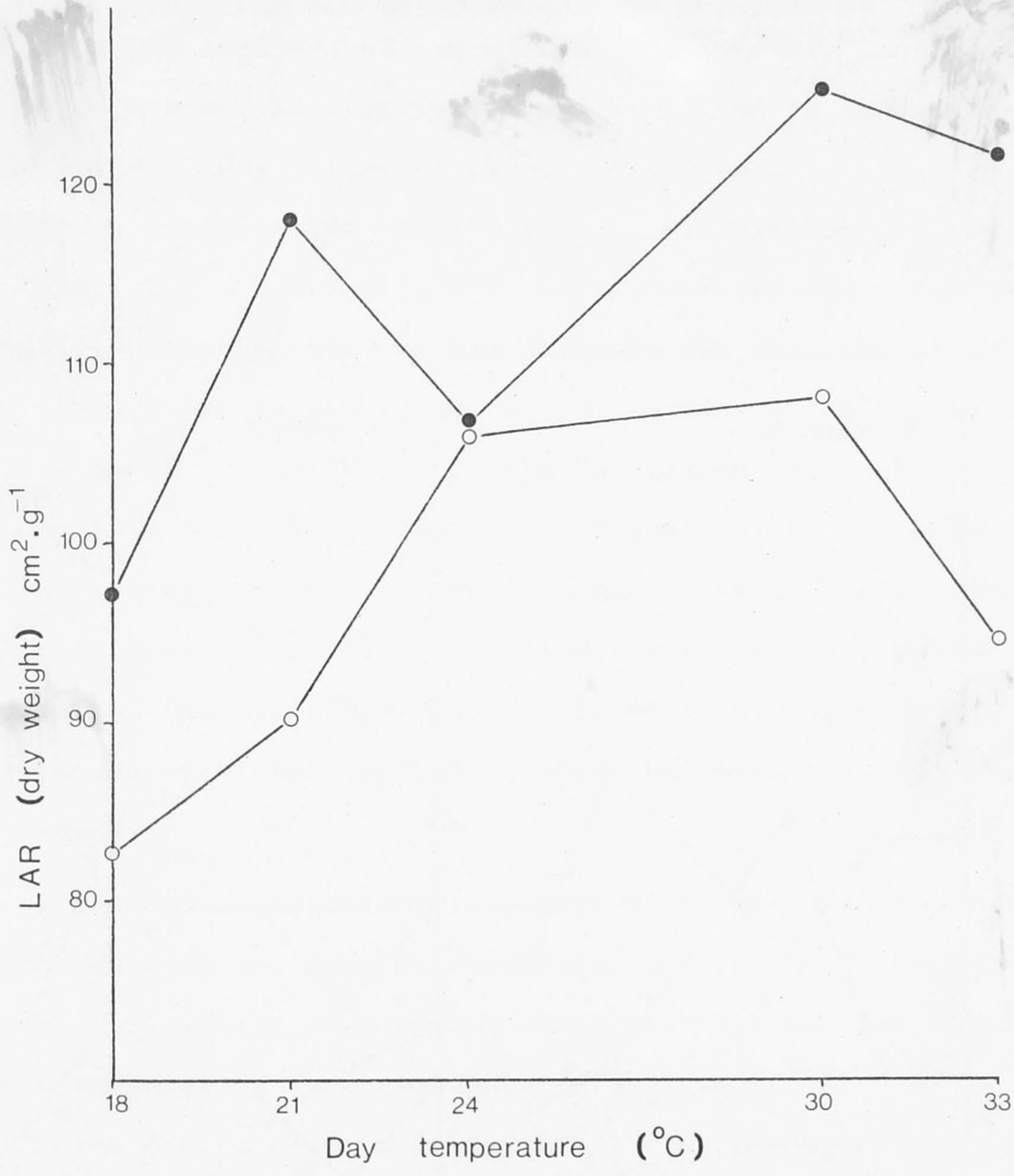
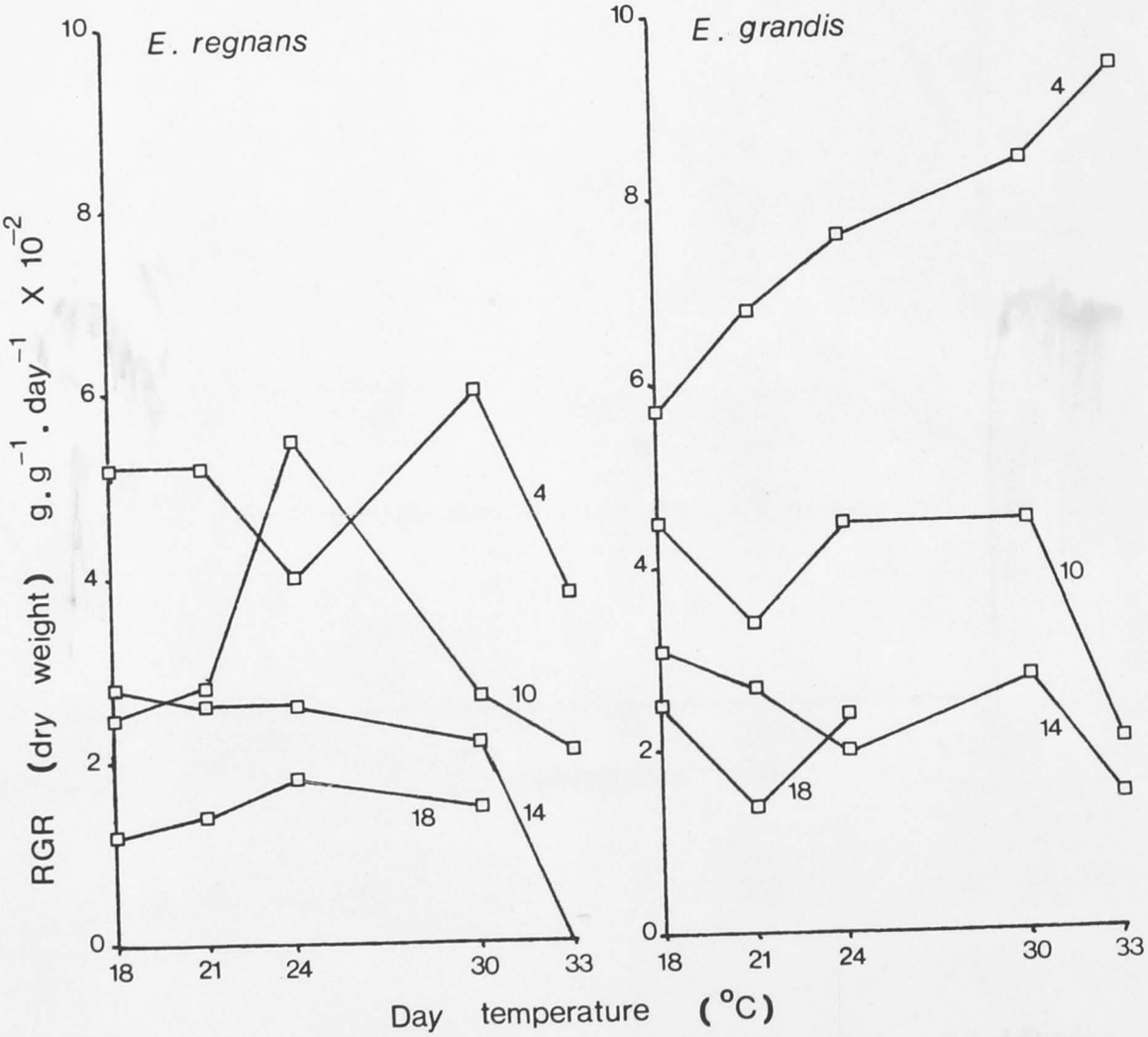


Figure 2-7. Leaf area ratio of seedlings of *E. regnans* (o) and *E. grandis* (●) after 10 weeks at different temperatures. Based on mean leaf area and dry weight data from 5 seedlings.

[1] The changes in RGR with age can be seen more clearly from the figure below.



Effects of temperature on relative dry weight growth rate of seedlings of *E. regnans* and *E. grandis* of different ages. The time since transfer in weeks is shown on each line.

[2] Data not presented here.

The effects of temperature on the components of LAR, namely leaf weight ratio (LWR) and specific leaf area (SLA) are shown in Figures 2-8 and 2-9. LWR is defined as the ratio of dry weight of leaves to dry weight of the whole plant, and SLA as the total leaf area divided by leaf dry weight. The variation in LWR with temperature is relatively small, so that effects on SLA dominate the response of LAR to temperature in both species. *E. regnans* has minimum LWR but maximum SLA at $24^{\circ}/19^{\circ}C$; the increasing leafiness (LWR) above this temperature leads to a higher LAR at $30^{\circ}/25^{\circ}C$, but is offset at $33^{\circ}/28^{\circ}C$ by the increased leaf thickness (lower SLA) so that LAR falls. The gradual decrease in LAR over the period of the experiment was due to decreasing LWR; SLA increased with seedling age, tending to level off after about 16 weeks.

The variations in growth of *E. grandis* with temperature and time are less straightforward. In the first 8 weeks after transfer to different temperature regimes, RGR increased with temperature over the whole range from $18^{\circ}/13^{\circ}C$ to $33^{\circ}/28^{\circ}C$. RGR also decreased with increasing age at all temperatures[1], but this decrease was much more rapid at $33^{\circ}/28^{\circ}C$, so that seedlings at this temperature had minimum RGR after 8 weeks. Mean RGR between 8 and 12 weeks (Figure 2-5) was equal at $24^{\circ}/19^{\circ}C$ and $30^{\circ}/25^{\circ}C$, suggesting an optimum temperature between these two; but between the 10 and 12 week harvests RGR at $30^{\circ}/25^{\circ}C$ increased and was still higher than at $24^{\circ}/19^{\circ}C$ between 12 and 16 weeks. *E. grandis* seedlings at $18^{\circ}/13^{\circ}C$ had higher RGR than those grown at $21^{\circ}/16^{\circ}C$ (Figure 2-5); this was true at all

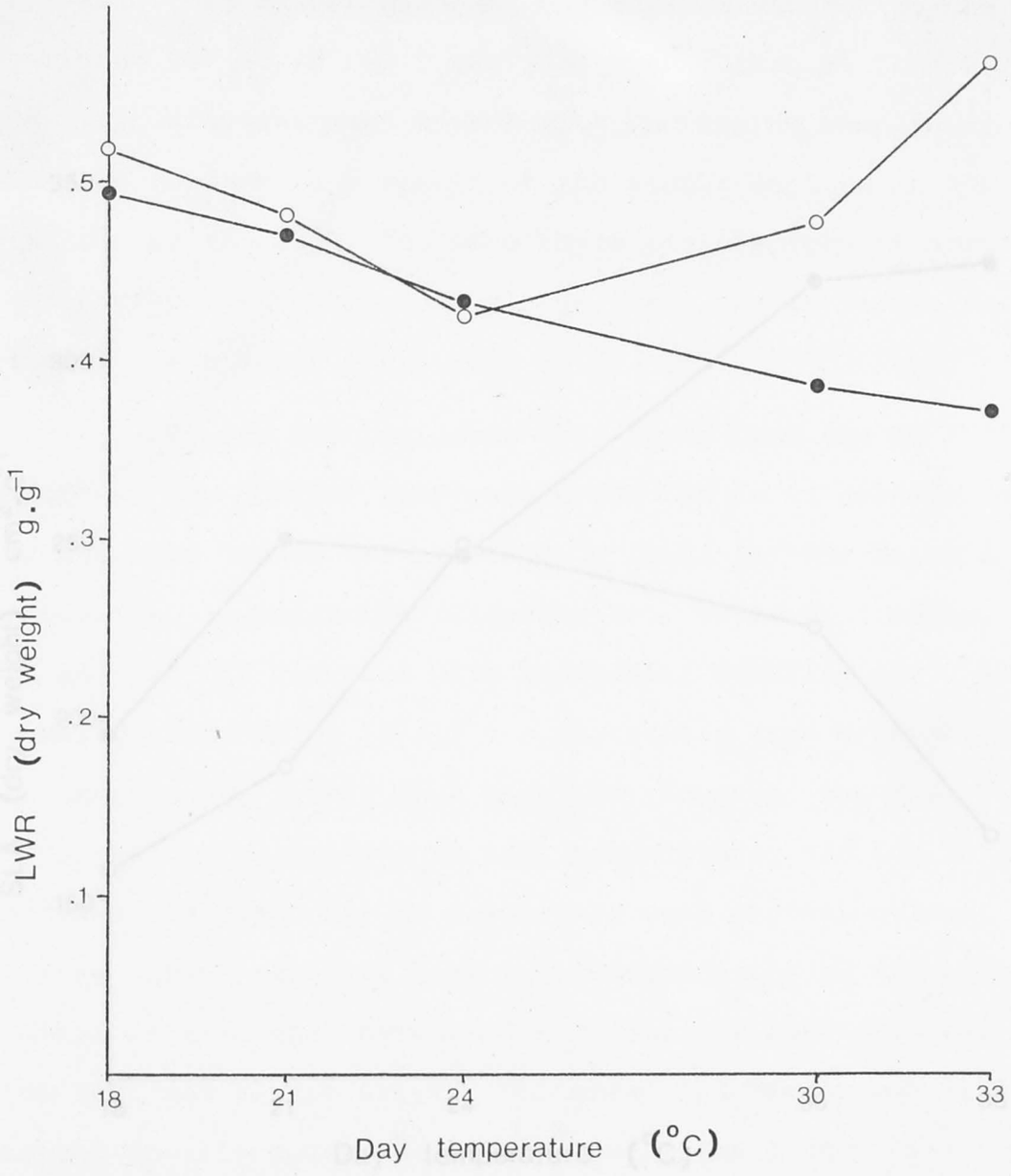


Figure 2-8. Leaf weight ratio of seedlings of *E. regnans* (o) and *E. grandis* (●) after 10 weeks at different temperatures. Based on mean dry weight data from 5 seedlings.

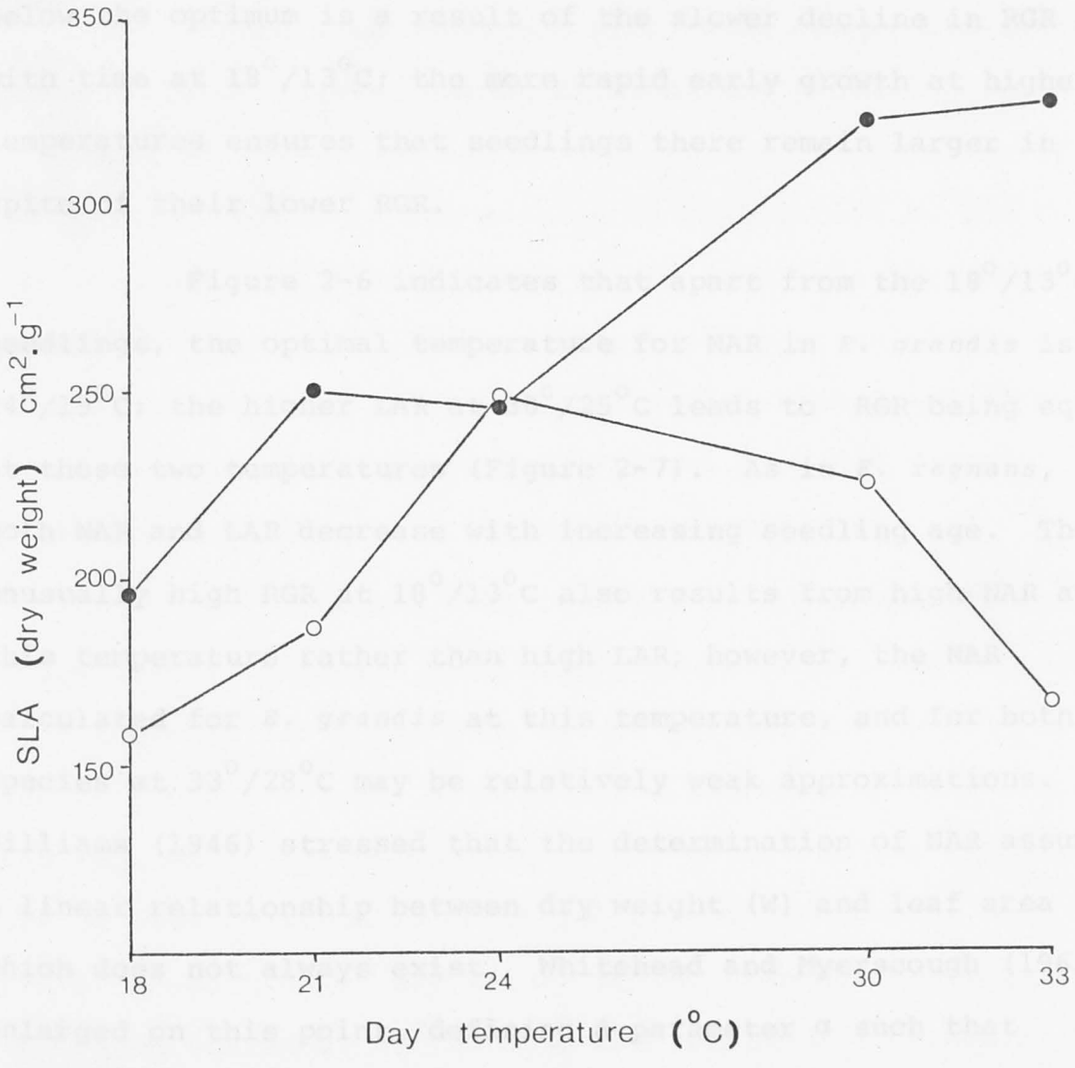


Figure 2-9. Specific leaf area of seedlings of *E. regnans* (o) and *E. grandis* (●) after 10 weeks at different temperatures. Based on mean leaf area and dry weight data from 5 seedlings.

times after the 8 week harvest, and towards the end of the experiment RGR at 18°/13°C was also higher than at 24°/19°C. This surprising increase in RGR with decreasing temperature below the optimum is a result of the slower decline in RGR with time at 18°/13°C; the more rapid early growth at higher temperatures ensures that seedlings there remain larger in spite of their lower RGR.

Figure 2-6 indicates that apart from the 18°/13°C seedlings, the optimal temperature for NAR in *E. grandis* is 24°/19°C; the higher LAR at 30°/25°C leads to RGR being equal at these two temperatures (Figure 2-7). As in *E. regnans*, both NAR and LAR decrease with increasing seedling age. The unusually high RGR at 18°/13°C also results from high NAR at this temperature rather than high LAR; however, the NAR calculated for *E. grandis* at this temperature, and for both species at 33°/28°C may be relatively weak approximations. Williams (1946) stressed that the determination of NAR assumes a linear relationship between dry weight (W) and leaf area (L) which does not always exist. Whitehead and Myerscough (1962) enlarged on this point, defining a parameter α such that $W = k.L^{\alpha} + c$, where k and c are constants. The values of α relevant to the NAR data of Figure 2-6 are as follows:-

	18°/13°C	21°/16°C	24°/19°C	30°/25°C	33°/28°C
E. grandis	1.46	1.04	1.04	1.11	4.73
E. regnans	1.01	0.94	1.12	1.01	1.36

The deviation of these values from unity must be considered important at 18°/13°C in *E. grandis*, and at

33°/28°C in both species.

2.3.3. Effects on dry weight distribution

Figure 2-10 shows the percentage contributions of stem and branch leaves, branches, stems and roots to total dry weight of *E. regnans* and *E. grandis* seedlings at the 16 week harvest. Most of the effects of temperature on the relative size of stem, branches and leaves have been mentioned already, either as qualitative observations or as growth analysis parameters, but it is worthwhile reiterating them briefly as part of a changing distribution of dry matter within the seedlings with increasing temperature.

In *E. grandis*, the fraction of dry weight stored in the leaves decreases with rising temperature. At temperatures above 18°/13°C the stem leaves account for only 3% or less of the dry weight, with little difference between temperature regimes. The percentage of dry weight in the branches is more or less constant up to 30°/25°C, then increases: the greater proportion of dry weight in the branches but less in the branch leaves at 33°/28°C is the result of a decrease in leaf size at this temperature rather than an increase in branch size, as mean dry weight per branch is lower than at 30°/25°C (Table 2-2).

Up to 30°/25°C, the decreasing contribution of the leaves of *E.grandis* to total dry weight is accompanied by an increase in the contribution from the stem, but this declines above 30°/25°C so it is apparent that at temperatures above the optimum a redistribution of growth occurs such that

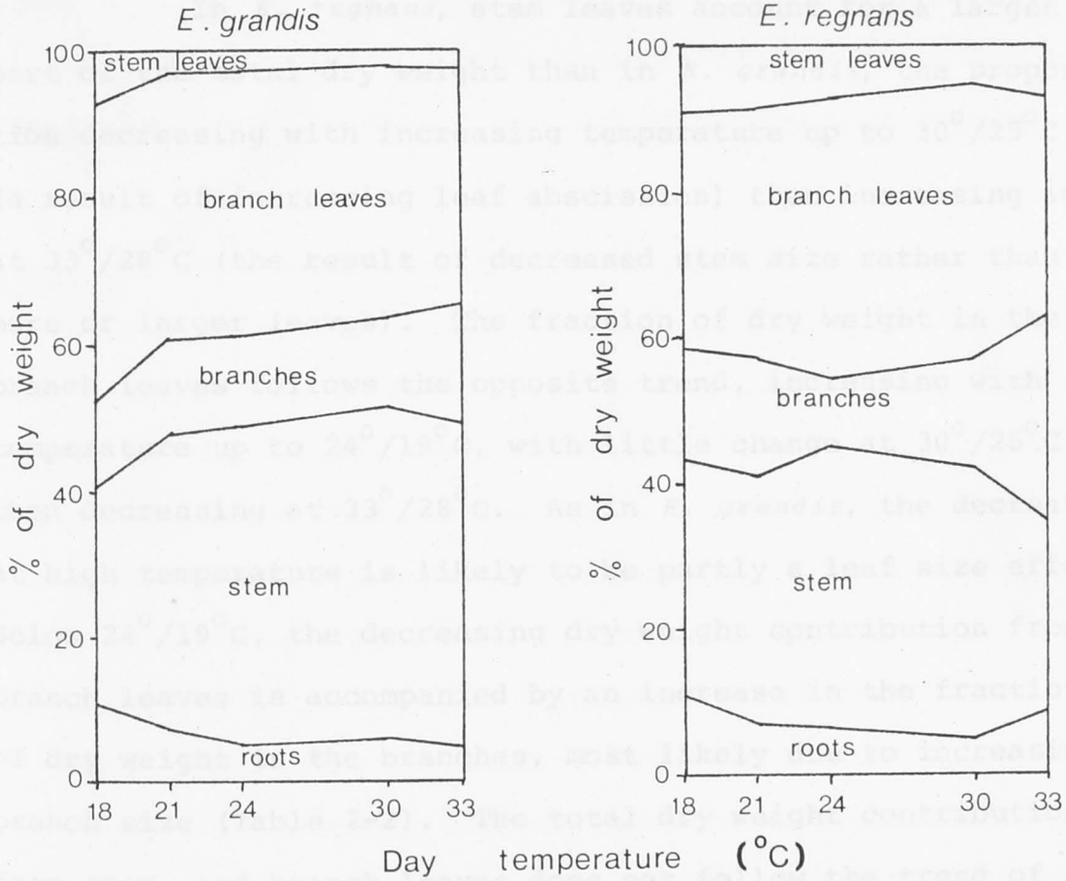


Figure 2-10. Dry weight distribution in seedlings of *E. regnans* (○) and *E. grandis* (●) after 16 weeks at different temperatures. (Mean of 5 seedlings).

branches increase in size at the expense of some stem growth. The increasing fraction of dry weight in the stem and branches is also accompanied by a decreasing percentage dry weight in the roots up to $24^{o}/19^{o}C$, above which little change occurs.

In *E. regnans*, stem leaves account for a larger part of the total dry weight than in *E. grandis*, the proportion decreasing with increasing temperature up to $30^{o}/25^{o}C$ (a result of increasing leaf abscission) then increasing again at $33^{o}/28^{o}C$ (the result of decreased stem size rather than more or larger leaves). The fraction of dry weight in the branch leaves follows the opposite trend, increasing with temperature up to $24^{o}/19^{o}C$, with little change at $30^{o}/25^{o}C$, then decreasing at $33^{o}/28^{o}C$. As in *E. grandis*, the decrease at high temperature is likely to be partly a leaf size effect. Below $24^{o}/19^{o}C$, the decreasing dry weight contribution from branch leaves is accompanied by an increase in the fraction of dry weight in the branches, most likely due to increasing branch size (Table 2-2). The total dry weight contribution from stem and branch leaves does not follow the trend of LWR described earlier (Figure 2-8): the 16 week harvest data displays a later stage in the decline of the seedlings at high temperatures, when the reduction in leaf size outweighs the effect of excessive branch growth which caused LWR to increase at high temperatures between 8 and 12 weeks.

The fraction of total dry weight in the branches of *E. regnans* seedlings at 16 weeks is minimal at $24^{o}/19^{o}C$, increasing strongly above this temperature to about 28% at $33^{o}/28^{o}C$. The $24^{o}/19^{o}C$ minimum may be a transient effect due

to recent branch abscission, as at both 12 and 20 weeks there is a steady increase in branch percentage dry weight from the lowest to the highest temperature regime. The stem percentage dry weight increases slightly up to $24^{\circ}/19^{\circ}C$, then decreases, especially above $30^{\circ}/25^{\circ}C$. Thus, branches increase in size at the expense of stem growth at supra-optimal temperatures exactly as in *E. grandis*, but the effect is more pronounced here.

The percentage of dry weight in the roots of *E. regnans* decreases with rising temperature up to $30^{\circ}/25^{\circ}C$, but increases at $33^{\circ}/28^{\circ}C$. This is not due to increased root growth at the highest temperature, but is a result of more rapid decline in stem and leaf growth than in root growth. The effects of temperature on the proportion of dry weight in the roots are commonly discussed in terms of root:shoot ratios, as plotted in Figure 2-11. This graph shows that up to $30^{\circ}/25^{\circ}C$, root:shoot ratios were very similar in both species at the 16 week harvest, despite the much more vigorous growth of *E. grandis* at the higher temperatures. The ratio shown for this species at $24^{\circ}/19^{\circ}C$ is slightly lower than at $30^{\circ}/25^{\circ}C$, but this is probably only a transient effect as at other harvests the root:shoot ratio decreased steadily with increasing temperature, and by 20 weeks the value at $24^{\circ}/19^{\circ}C$ had increased while those at other temperatures continued to decrease.

The root:shoot ratio of *E. regnans* at $33^{\circ}/28^{\circ}C$ is much greater than shown by this species at $30^{\circ}/25^{\circ}C$, or by *E. grandis* at $33^{\circ}/28^{\circ}C$, but Figure 2-12 shows that root dry weight at the highest temperature is well below that at other

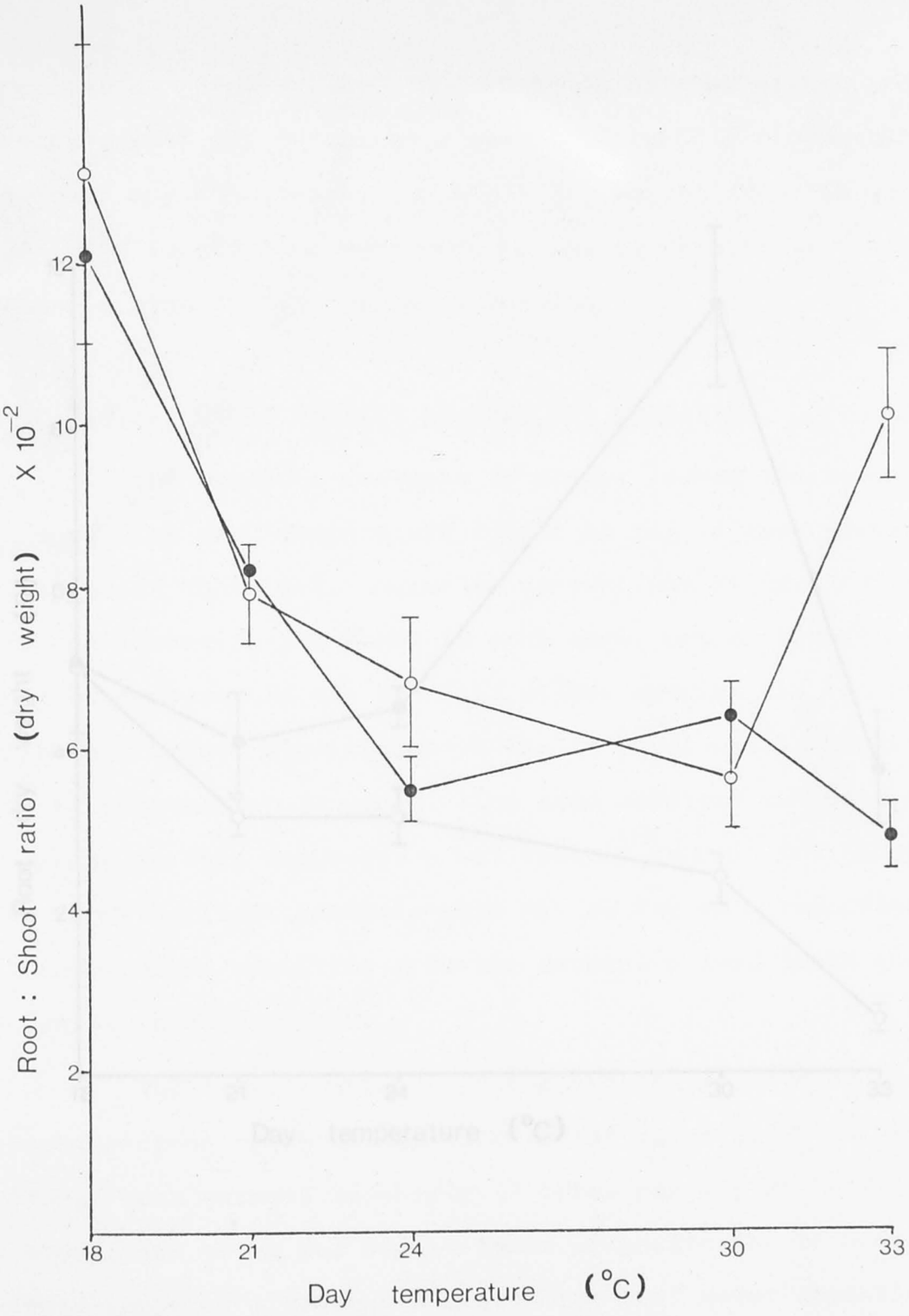


Figure 2-11. Ratio of root to shoot dry weight of *E. regnans* (○) and *E. grandis* (●) seedlings after 16 weeks at different temperatures. Each point is the mean of 5 seedlings, ± standard error.

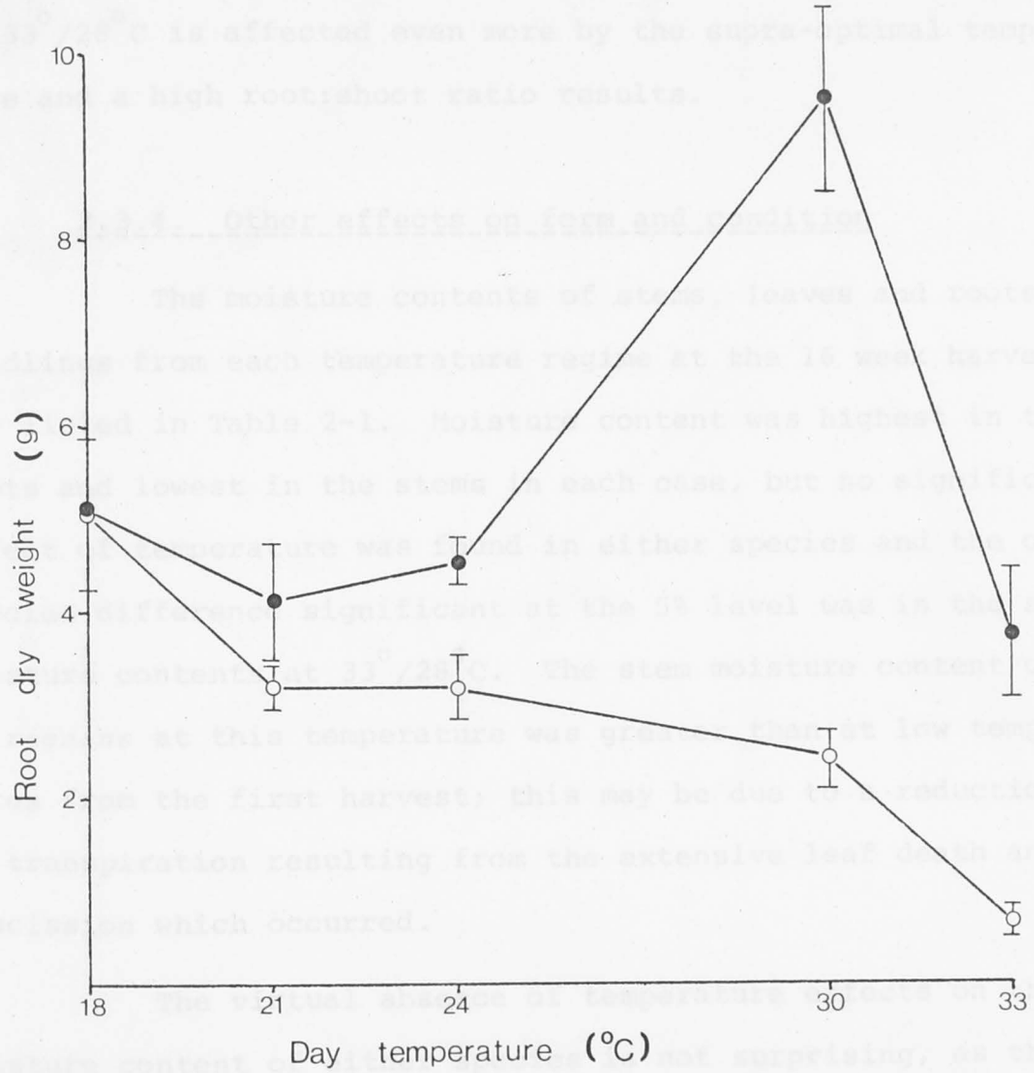


Figure 2-12. Dry weight of roots of *E. regnans* (○) and *E. grandis* (●) seedlings after 16 weeks at different temperatures. Each point is the mean of 5 seedlings, ± standard error.

temperatures. However, due to cessation of stem growth and decreasing leaf dry weight as a result of rapidly progressing leaf death and abscission, the shoot dry weight after 16 weeks at $33^{o}/28^{o}C$ is affected even more by the supra-optimal temperature and a high root:shoot ratio results.

2.3.4. Other effects on form and condition

The moisture contents of stems, leaves and roots of seedlings from each temperature regime at the 16 week harvest are listed in Table 2-1. Moisture content was highest in the roots and lowest in the stems in each case, but no significant effect of temperature was found in either species and the only species difference significant at the 5% level was in the stem moisture contents at $33^{o}/28^{o}C$. The stem moisture content of *E. regnans* at this temperature was greater than at low temperatures from the first harvest; this may be due to a reduction in transpiration resulting from the extensive leaf death and abscission which occurred.

The virtual absence of temperature effects on the moisture content of either species is not surprising, as the seedlings were watered regularly (3 times per day at $30^{o}/25^{o}C$ and above, and twice per day at lower temperatures) in order to avoid imposing a drought stress. The leaf water potential data obtained (Figure 2-13) suggest that small differences between temperatures may have resulted from the watering regime: leaf water potential of both species decreases as day temperature increases from 18^{o} to $24^{o}C$, then increases sharply at $30^{o}C$ before decreasing again with a further rise in tempera-

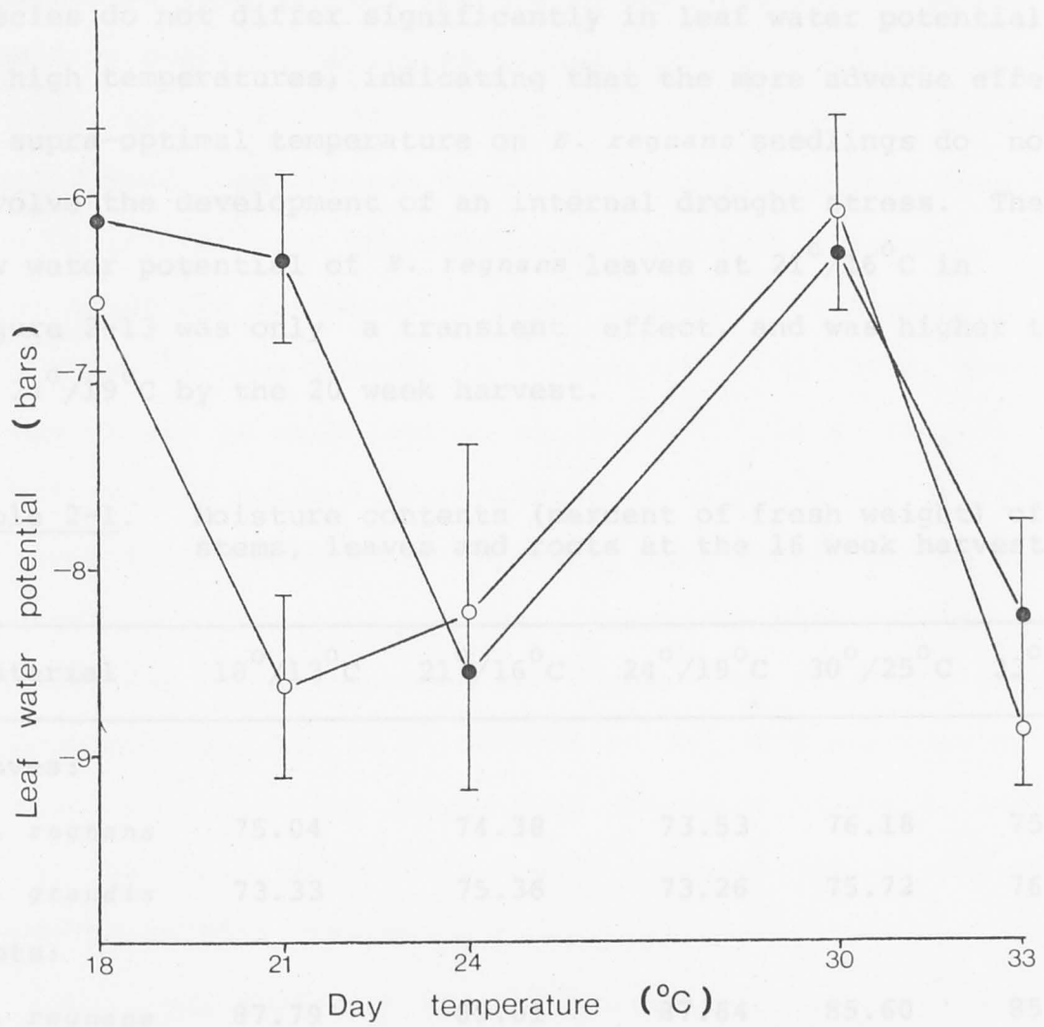


Figure 2-13. Water potential of young leaves of *E. regnans* (○) and *E. grandis* (●) seedlings after 16 weeks at different temperature regimes. Each point is the mean of 5 seedlings, ± standard error.

ture. The extra daily watering applied at 30°C and above appears to have interrupted a steadily decreasing trend of water potential with increasing temperature. However, the two species do not differ significantly in leaf water potential at high temperatures, indicating that the more adverse effects of supra-optimal temperature on *E. regnans* seedlings do not involve the development of an internal drought stress. The low water potential of *E. regnans* leaves at 21°/16°C in Figure 2-13 was only a transient effect, and was higher than at 24°/19°C by the 20 week harvest.

Table 2-1. Moisture contents (percent of fresh weight) of stems, leaves and roots at the 16 week harvest.

Material	18°/13°C	21°/16°C	24°/19°C	30°/25°C	33°/28°C
Leaves:					
E. regnans	75.04	74.38	73.53	76.18	75.36
E. grandis	73.33	75.36	73.26	75.72	76.05
Roots:					
E. regnans	87.79	89.01	87.64	85.60	85.33
E. grandis	89.09	88.21	89.35	87.80	89.11
Stems:					
E. regnans	69.36	70.54	70.35	69.87	73.64
E. grandis	71.03	69.83	67.80	67.65	66.38

It has been seen (Figures 2-1 to 2-4) that the growth of *E. regnans* was slower than that of *E. grandis* at all

temperatures. In Figure 2-14 the number of stem leaf pairs differentiated by each species up to the 16 week harvest is plotted against temperature, revealing that the difference in seedling size between species is due to a lesser number of stem internodes and branches rather than an equal number of smaller size in *E. regnans*; that is, the process of development of seedlings towards maturity is slower in this species. This correlates with the difference observed in rates of germination and formation of first leaves. Development in this sense is thus fastest in *E. grandis* at $33^{o}/28^{o}$C, even though growth is much faster at $30^{o}/25^{o}$C. In *E. regnans*, the number of leaf pairs differentiated at $24^{o}/19^{o}$C and $30^{o}/25^{o}$C differed little even after 20 weeks, when growth at the higher temperature was falling behind. It appears that optimal temperatures for development are higher than those for growth in both species, but in *E. regnans* at $33^{o}/28^{o}$C the rate of initiation of new stem leaves fell off after 12 weeks, indicating that this temperature regime is supra-optimal for both processes in this species.

The different temperature optima for stem leaf initiation and for growth in height and dry weight suggest that differences in internode length, branch size and stem diameter may exist between species and temperature regimes. While these parameters were not measured directly, they can be assessed by indices derived from the dry weight, height and leaf number data. These are shown in Table 2-2, for seedlings at the 16 week harvest.

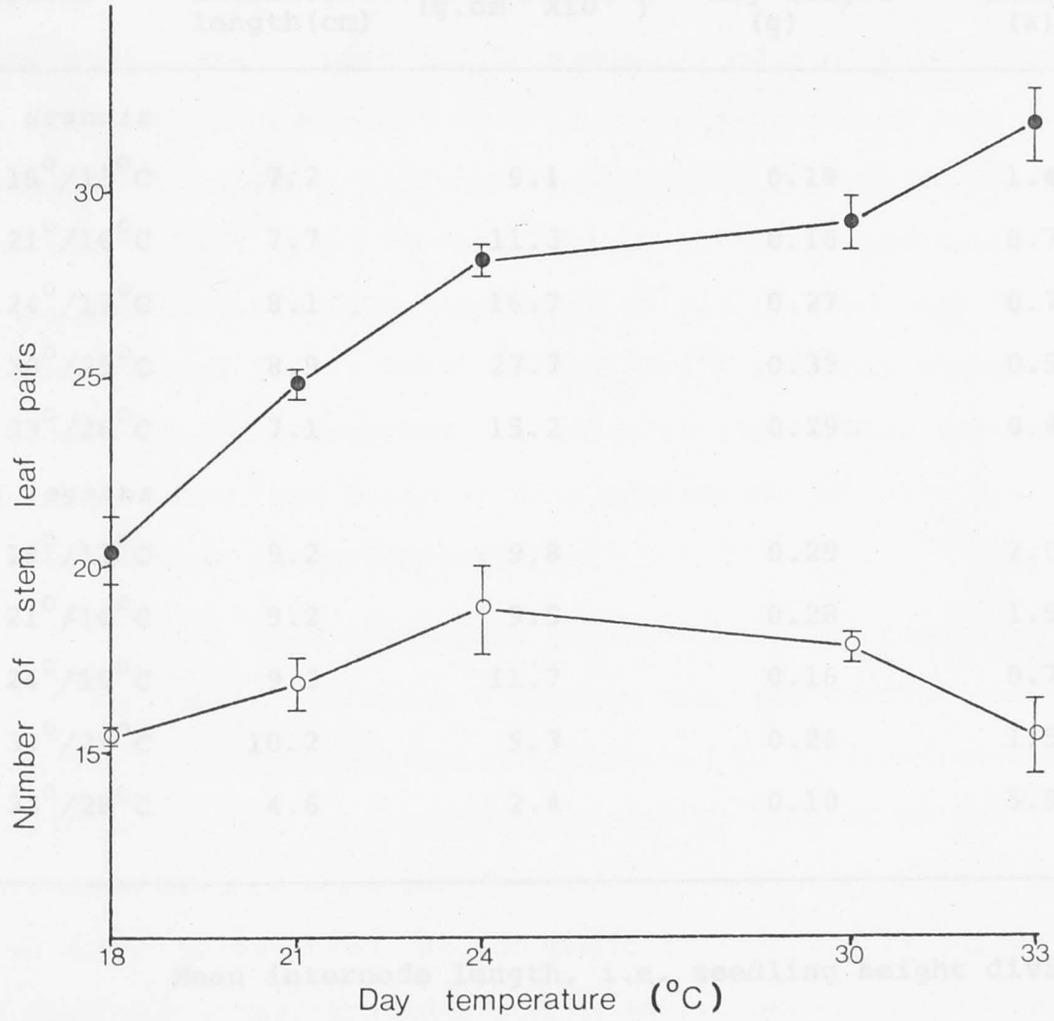


Figure 2-14. Numbers of stem leaf pairs differentiated by seedlings of *E. regnans* (○) and *E. grandis* (●) after 16 weeks at different temperature regimes. (Mean of 5 seedlings at each temperature, ± standard error).

Table 2-2. Indices of stem and branch size in *E. regnans* and *E. grandis* seedlings at the 16 week harvest.

Seedling source	Mean internode length (cm)	Cambial growth ($g.cm^{-1} X10^{-2}$)	Mean branch dry weight (g)	Branch:stem ratio (%)
E. grandis				
18°/13°C	7.2	9.1	0.19	1.44
21°/16°C	7.7	11.3	0.16	0.72
24°/19°C	8.1	16.7	0.27	0.70
30°/25°C	8.9	27.7	0.39	0.54
33°/28°C	7.1	15.2	0.29	0.86
E. regnans				
18°/13°C	9.2	9.8	0.29	2.08
21°/16°C	9.2	9.5	0.28	1.92
24°/19°C	9.3	11.7	0.16	0.76
30°/25°C	10.2	9.3	0.26	1.52
33°/28°C	4.6	2.4	0.10	5.98

Mean internode length, i.e. seedling height divided by the number of stem leaf pairs, increases with temperature in both species to a maximum at 30°/25°C, then falls sharply in *E. regnans* and less so in *E. grandis*. The decline in internode length with decreasing temperature below 30°/25°C is more rapid in *E. grandis*. At all temperatures except 33°/28°C, internodes of *E. regnans* were longer, reinforcing the point made above that the smaller size of these seedlings was a result of slower development. By the 20 week harvest, mean internode length of this species was greatest at 24°/19°C,

demonstrating that a reduction in internode length is a further sign of the onset of high temperature stress in *E. regnans*.

The cambial growth index of Table 2-2 is the quotient of stem dry weight and height, and therefore measures mean stem diameter and density combined, both of which depend largely on the number and size of cells produced by the cambium. This index is greatest at $30^{\circ}/25^{\circ}$C in *E. grandis*, and at $24^{\circ}/19^{\circ}$C in *E. regnans*, values in the latter species being considerably lower except at $18^{\circ}/13^{\circ}$C. The very low value obtained for *E. regnans* at $33^{\circ}/28^{\circ}$C reflects the extreme weakness of stems observed, and even at the 16 week harvest it can be seen that stems of this species at $30^{\circ}/25^{\circ}$C are weaker than at lower temperatures.

The mean dry weight per branch of the seedlings in Table 2-2 appears to reach a maximum near the optimum temperature for growth of each species but also to increase at low temperatures, and is therefore apparently subject to the influence of more than one factor. A correlation with stem size might be expected, so the ratio of mean branch dry weight to stem dry weight provides a more useful measure of branch size. The data of Table 2-2 indicate that this ratio declines with increasing temperature up to the optimum ($30^{\circ}/25^{\circ}$C in *E. grandis*, $24^{\circ}/19^{\circ}$C in *E. regnans*), then increases. The relative branch size in the latter species is greater at all temperatures, reaching almost 6% of stem size at $33^{\circ}/28^{\circ}$C; again the effect of supra-optimal temperature on *E. regnans* was clearly present at $30^{\circ}/25^{\circ}$C as early as 16 weeks after transfer.

2.4. Discussion

The large body of data collected in the growth study and discussed in the preceding sections has indicated important differences between the two species studied in the effects of temperature on seedling growth and development. These differences are of two types: differences in optimum temperatures, and in the response to temperatures above and below optimum values. Without referring to individual parameters, whose optimum temperatures are not always the same within either species, it is obvious that the optimum temperature for growth and development of *E. grandis* is higher than that of *E. regnans*. The difference in optima during the early period of growth studied is however not very large; an analogous study by Eagles (1967a) comparing two climatic races of *Dactylis glomerata* demonstrated much more dramatic differences in the effects of temperature on such parameters as dry weight distribution and root:shoot ratio between plants adapted to a cool climate and those from a warm climate. The more subtle differences observed between the two eucalypts in this study probably result from a smaller difference in optimum temperatures.

The decrease in optimum temperature with increasing age of *E. regnans* seedlings reported by Eldridge (1969) and clearly demonstrated here causes the gap between species to become wider with time. Although growth of *E. grandis* in the first few weeks after transfer from $24^{o}/19^{o}C$ was fastest at $33^{o}/28^{o}C$, no evidence was found to suggest a continuing downward trend in the optimum temperature for growth of this species as seen in *E. regnans*. It is not unusual for plants

to display different temperature optima during different stages of development (e.g. Hartsema 1961), and the initial high optimum in *E. grandis* (and perhaps *E. regnans*) can be seen as an example of this phenomenon.

The difference between species in gradient of the response to temperatures above and below optimum is as important as the difference in the actual optima. $33^{o}/28^{o}$C is certainly supra-optimal for both species, and similar responses in leaf and branch morphology occur; however, the effects on *E. regnans* are much more pronounced, and accompanied by other symptoms which eventually lead to seedling death. On the other hand *E. grandis* seedlings at $18^{o}/13^{o}$C were in several ways more affected by sub-optimal temperature than *E. regnans*, even in the early part of the experiment when the difference in optimum temperatures of the two species was small.

The growth study has thus demonstrated a difference in optimum temperature for growth of each species, and a decline in the optimum for *E. regnans* with time. These effects are the same as postulated in the previous Chapter to account for the failure of *E. regnans* and other species in the Coffs Harbour species trials, as described by Pryor (1972). Furthermore, the seedlings displayed disordered growth of stem and branches before they died, as mentioned by Pryor and resembling symptoms observed by this author on a few surviving trees of *E. sieberi* and *E. st. johnii* during an inspection of the Coffs Harbour trial site in 1974.

The essential difference between the effects of supra-optimal temperature on the growth of *E. regnans* as displayed in the phytotron study and the growth of this species

observed at Coffs Harbour was in the time span over which the effects took place: growth in the field was maintained for two or three years before the saplings died, while at $30^{\circ}/25^{\circ}C$ in the glasshouse healthy growth lasted only about 16 weeks. This difference can be readily accounted for in terms of temperature differences between field and glasshouse. In field conditions temperature varies continuously, with seasonal as well as diurnal variations. Thus, even though day temperature may exceed $30^{\circ}C$ on a number of occasions during the year, the phytotron temperature regime of $30^{\circ}C$ every day and $25^{\circ}C$ every night is far harsher than would be experienced in the field. *E. regnans* seedlings in the glasshouses showed increasing periods of healthy growth prior to decline, with decreasing temperature: less than 3 weeks at $33^{\circ}/28^{\circ}C$, about 16 weeks at $30^{\circ}/25^{\circ}C$ and about 30 weeks at $24^{\circ}/19^{\circ}C$. Therefore, in the relatively mild (but still supra-optimal) climate of Coffs Harbour a period of two years or more before decline is not unreasonable.

An objective of the growth study was to attempt to simulate the differences in growth of *E. regnans* and *E. grandis* displayed at Coffs Harbour, in controlled environments. The data presented in this Chapter indicate that many of the phenomena exhibited by *E. regnans* at Coffs Harbour are repeated after a relatively short growth period at higher temperatures. In consequence, some possible physiological causes of the differences in growth of *E. regnans* and *E. grandis* seedlings were examined at a range of temperatures, with other environmental variables held constant.

CHAPTER 3

EFFECTS OF TEMPERATURE ON PHOTOSYNTHESIS AND DARK RESPIRATION RATES IN *E. REGNANS* AND *E. GRANDIS* SEEDLINGS

3.1. Introduction

It has been shown that a difference in the growth responses of *E. regnans* and *E. grandis* seedlings to temperature exists under controlled conditions, which may be sufficient to account for the difference in performance of the two species observed in field trials. An examination of some of the physiological factors underlying the effects on growth and morphology described in the previous Chapter is now required, as a first step towards understanding the mechanisms which lead to death of one species at temperatures favourable for growth of the other.

Growth analysis (Section 2.3.2.) has indicated that the differences in relative growth rate (RGR) between species and day/night temperature regimes are ascribable largely to differences in net assimilation rate (NAR; see Figure 2-6). As the rate of dry matter accumulation by a plant depends on the rates of photosynthesis and respiration maintained, an investigation of these processes in each species over a range of temperatures might be expected to reveal important differences which could help to explain the observed differences in growth. Even if a correlation between the effects of tempera-

ture on gas exchange and on growth rates cannot be found, information on the variation in rates of photosynthesis and respiration with temperature in *E. grandis* and *E. regnans* would be a useful extension to the data collected in the growth study (Chapter 2), in delineating the difference in temperature responses of the two species. Such a lack of relationship between NAR and photosynthetic rate was reported by Treharne and Eagles (1970), although in earlier experiments (Eagles and Treharne 1969) high correlations had been found; only in the earlier work was RGR strongly correlated with NAR (rather than leaf area ratio), as observed in the eucalypt seedlings of this study.

While it is possible to measure gas exchange rates of whole seedlings by enclosing them in large assimilation chambers, the rapid growth of eucalypt seedlings in the phytotron makes such an approach feasible only for very young material. As some important growth differences between species and temperature regimes did not develop until 8 weeks or longer after the transfer of plants to different temperatures, measurements of respiration rates were made on leaf buds and root tips, and photosynthesis was measured using single attached leaves. The data obtained may provide a better indication of the effects of temperature on these processes than would data from whole-plant studies, in which it is difficult to prevent other factors such as differences in seedling morphology from influencing the results.

3.2. Materials and methods

3.2.1. Plant materials

Measurements of respiration rates of leaf buds and root tips were carried out on the seedlings harvested for the growth study described in Chapter 2. Cultural conditions and harvesting procedures were entirely as set out in Section 2.2.

For the measurements of net photosynthesis a second batch of *E. grandis* and *E. regnans* seedlings was raised in the phytotron glasshouses. As before, seeds were germinated at $24^{o}/19^{o}C$ in 1:1 perlite/vermiculite. *E. regnans* seedlings were potted 7 weeks after sowing, when two leaf pairs had developed, and *E.grandis* 4 weeks after sowing, with three leaf pairs present. Two seedlings were planted in each pot, but after a further 2 weeks at $24^{o}/19^{o}C$ the seedlings were thinned to one per pot, discarding unusually large or small seedlings to leave an essentially uniform population of each species. Four seedlings of each species were then transferred to glasshouses at $18^{o}/13^{o}C$, $21^{o}/16^{o}C$, $24^{o}/19^{o}C$, $27^{o}/22^{o}C$, $30^{o}/25^{o}C$ and $33^{o}/28^{o}C$. Conditions of watering, nutrient supply, photoperiod and thermoperiod were as described in Section 2.2.

Unlike the determination of respiration rates, the measurements of photosynthesis were confined to a comparison of the effects of temperature on each species, with no study of the change in photosynthesis with seedling age. Both the stage of development of seedlings (number of stem leaves differentiated) and the maturity of individual leaves are known to affect photosynthesis (McPherson and Slatyer 1972); measurements were therefore made at a definite stage of develop-

ment rather than at a fixed time after transfer to the different temperature regimes. The 15 leaf pair stage was chosen as a representative time to make these measurements; the relation between stage of development and time since transfer for the plants involved is shown in Figure 3-1, where it can be seen that *E. regnans* seedlings grown at $30^{\circ}/25^{\circ}C$ reached 15 leaf pairs after about 16 weeks, when the decline in growth rate was just beginning. The rate of net photosynthesis of the youngest fully expanded stem leaf pair was measured: in nearly every case this was the twelfth pair.

3.2.2. Measurement of respiration

The terminal buds from stems and branches, and healthy root tips (2-4 cm in length)were removed from the 5 seedlings randomly selected for each harvest. Six 0.05 g samples of leaf buds and six 0.15 g samples of root tips were then weighed out and placed in small Warburg flasks with 0.5 ml of distilled water. The centre wells of three leaf and three root flasks contained a filter paper wick bathed in 0.2 ml of 10% KOH solution.

The flasks were connected to manometers and allowed to equilibrate for one hour in a Warburg apparatus (Umbreit *et al.* 1964), with the water bath maintained at the day temperature at which the harvested plants had been grown. Two empty flasks were included, to act as thermobarometers. Respiration was allowed to proceed for 3 hours after equilibration, the change in pressure in each flask being measured every 30 minutes at constant gas volume as a check that respiration was

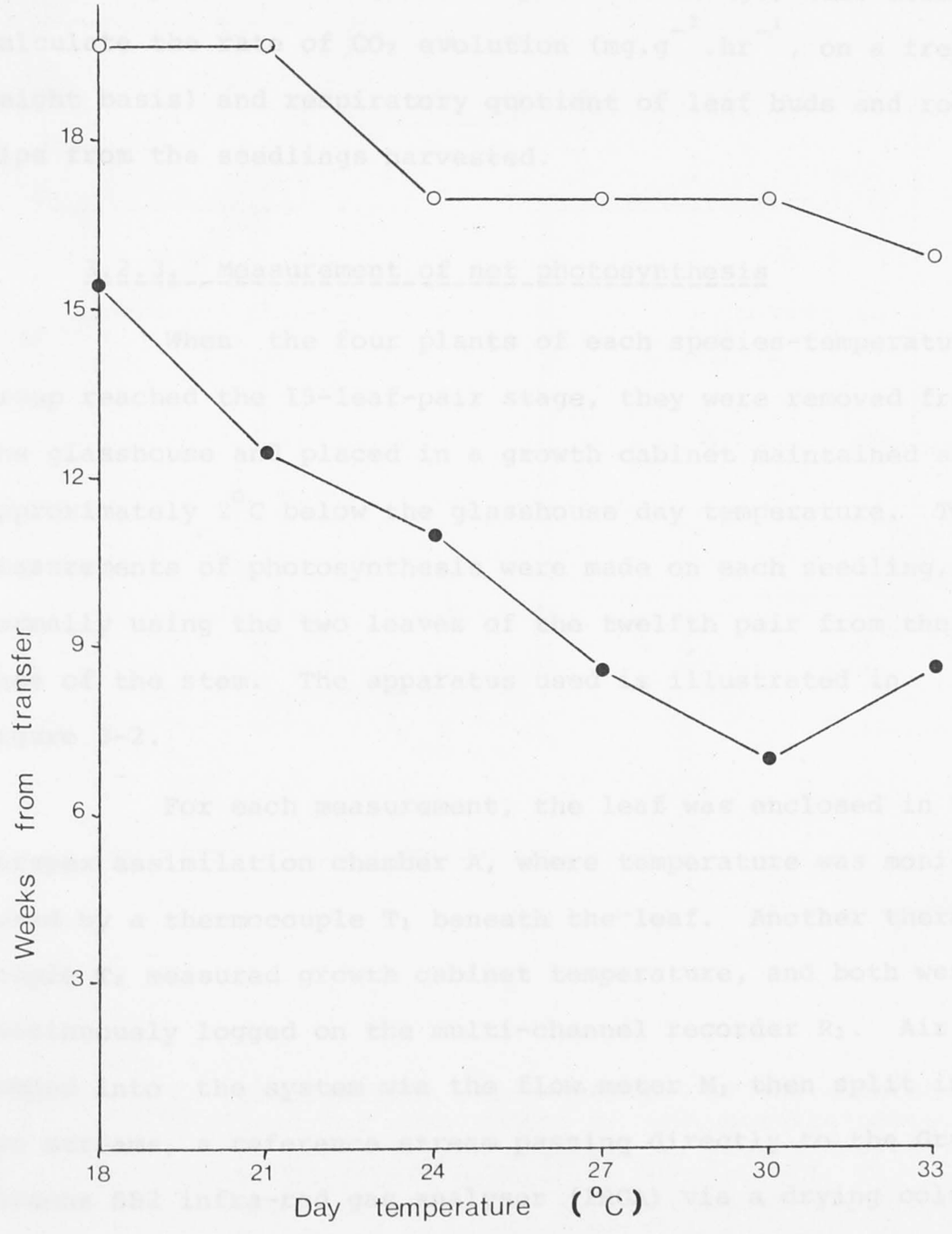


Figure 3-1. Time taken by seedlings of *E. regnans* (○) and *E. grandis* (●) to reach the 15 leaf-pair stage at different temperatures.

proceeding at a steady rate. The total pressure change over 3 hours, corrected for changes in temperature and atmospheric pressure by the thermobarometer pressure change, were used to calculate the rate of CO_2 evolution ($mg.g^{-1}.hr^{-1}$, on a fresh weight basis) and respiratory quotient of leaf buds and root tips from the seedlings harvested.

3.2.3. Measurement of net photosynthesis

When the four plants of each species-temperature group reached the 15-leaf-pair stage, they were removed from the glasshouse and placed in a growth cabinet maintained at approximately $2^{o}C$ below the glasshouse day temperature. Two measurements of photosynthesis were made on each seedling, normally using the two leaves of the twelfth pair from the base of the stem. The apparatus used is illustrated in Figure 3-2.

For each measurement, the leaf was enclosed in the Perspex assimilation chamber A, where temperature was monitored by a thermocouple T_1 beneath the leaf. Another thermocouple T_2 measured growth cabinet temperature, and both were continuously logged on the multi-channel recorder R_1. Air was pumped into the system via the flow meter M_1 then split into two streams, a reference stream passing directly to the Grubb-Parsons SB2 infra-red gas analyser (IRGA) via a drying column D_1 and flow meter M_4 while the other stream was passed across the leaf in the assimilation chamber before flowing to the IRGA. The flow rate to the chamber was maintained at 3.0 $l.min^{-1}$ by

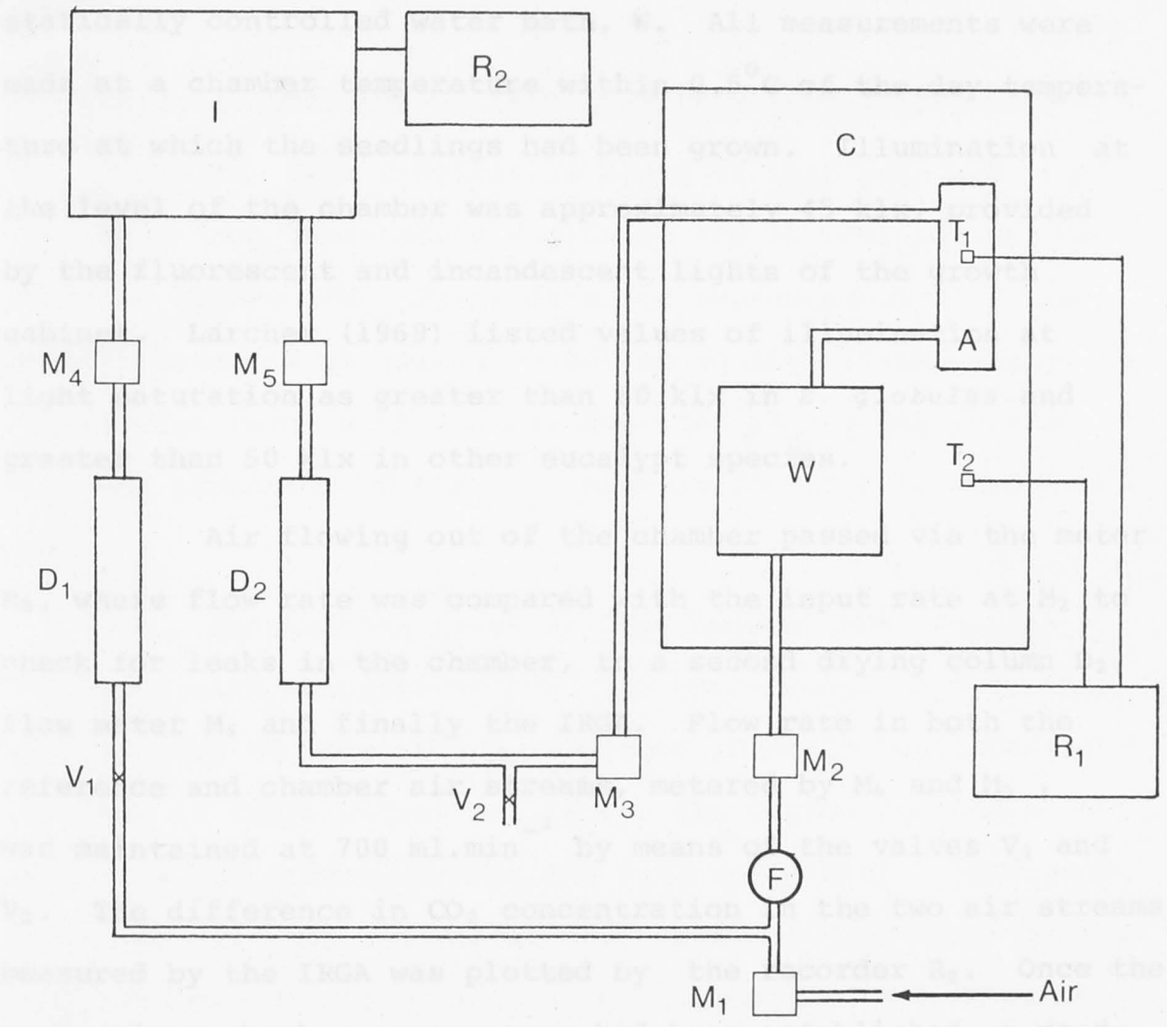


Figure 3-2. Diagrammatic representation of the apparatus used for measurement of net photosynthesis. A, assimilation chamber; C, growth cabinet; W, waterbath containing copper coil; T_1, T_2, thermocouples; R_1, R_2, recorders; M_1, M_2, M_3, M_4, M_5, flow meters; F, flow control; V_1, V_2, valves; D_1, D_2, drying columns; I, infra-red gas analyser.

means of the flow control F and meter M_2, and additional control over the chamber temperature was obtained by passing the input air stream through a copper coil immersed in a thermostatically controlled water bath, W. All measurements were made at a chamber temperature within 0.5°C of the day temperature at which the seedlings had been grown. Illumination at the level of the chamber was approximately 45 klx, provided by the fluorescent and incandescent lights of the growth cabinet. Larcher (1969) listed values of illumination at light saturation as greater than 60 klx in *E. globulus* and greater than 50 klx in other eucalypt species.

Air flowing out of the chamber passed via the meter M_3, where flow rate was compared with the input rate at M_2 to check for leaks in the chamber, to a second drying column D_2, flow meter M_5 and finally the IRGA. Flow rate in both the reference and chamber air streams, metered by M_4 and M_5, was maintained at 700 $ml.min^{-1}$ by means of the valves V_1 and V_2. The difference in CO_2 concentration in the two air streams measured by the IRGA was plotted by the recorder R_2. Once the appropriate chamber temperature had been established, a reading was taken as soon as the recorder indicated that steady state photosynthesis was proceeding. The leaf was then removed from the assimilation chamber and its fresh weight and area were measured; often the leaf was too long to fit completely in the chamber used, so the part of the leaf which had been enclosed in the chamber was cut off and measured. The enclosed leaf area was usually close to 20 cm^2. The rate of net photosynthesis of each leaf was calculated from the IRGA reading,

the flow rate and the leaf area or leaf weight measurements, in mg $CO_2.dm^{-2}.hr^{-1}$ or mg $CO_2.g^{-1}.hr^{-1}$.

3.3. Results

3.3.1. Respiration rates and respiratory quotients

The dark respiration rates of leaf buds and root tips from each temperature regime at the 16 week harvest are shown in Figure 3-3. These data are representative of the results obtained at all harvests, as no significant change in respiration rates with age was detected at any temperature, with the exception of the root tips at 33°/28°C as discussed below.

Respiration rates of leaf buds of both species increased with temperature over the whole range tested; this was expected as respiration in plants commonly continues to increase at temperatures well above the optima for photosynthesis and growth (Kramer and Kozlowski 1960). Leaf buds of *E. regnans* respired faster than those of *E. grandis* at low temperatures but slower at high temperatures; estimates of Q_{10} for the two species in the temperature range 20-30°C, obtained from smoothed curves drawn from the data of Figure 3-3, were 1.77 for *E. grandis* and 1.34 for *E. regnans*. Although the difference in respiration rates between the species is not very great even at 33°/28°C, the lower respiration rate of *E. regnans* seedlings at high temperatures may constitute an advantage for this species. This result is in contrast with the findings of Eagles (1967b) with races of *Lolium perenne* from different thermal environments, where plants from the warmer climate showed slower respiration (and more rapid photo-

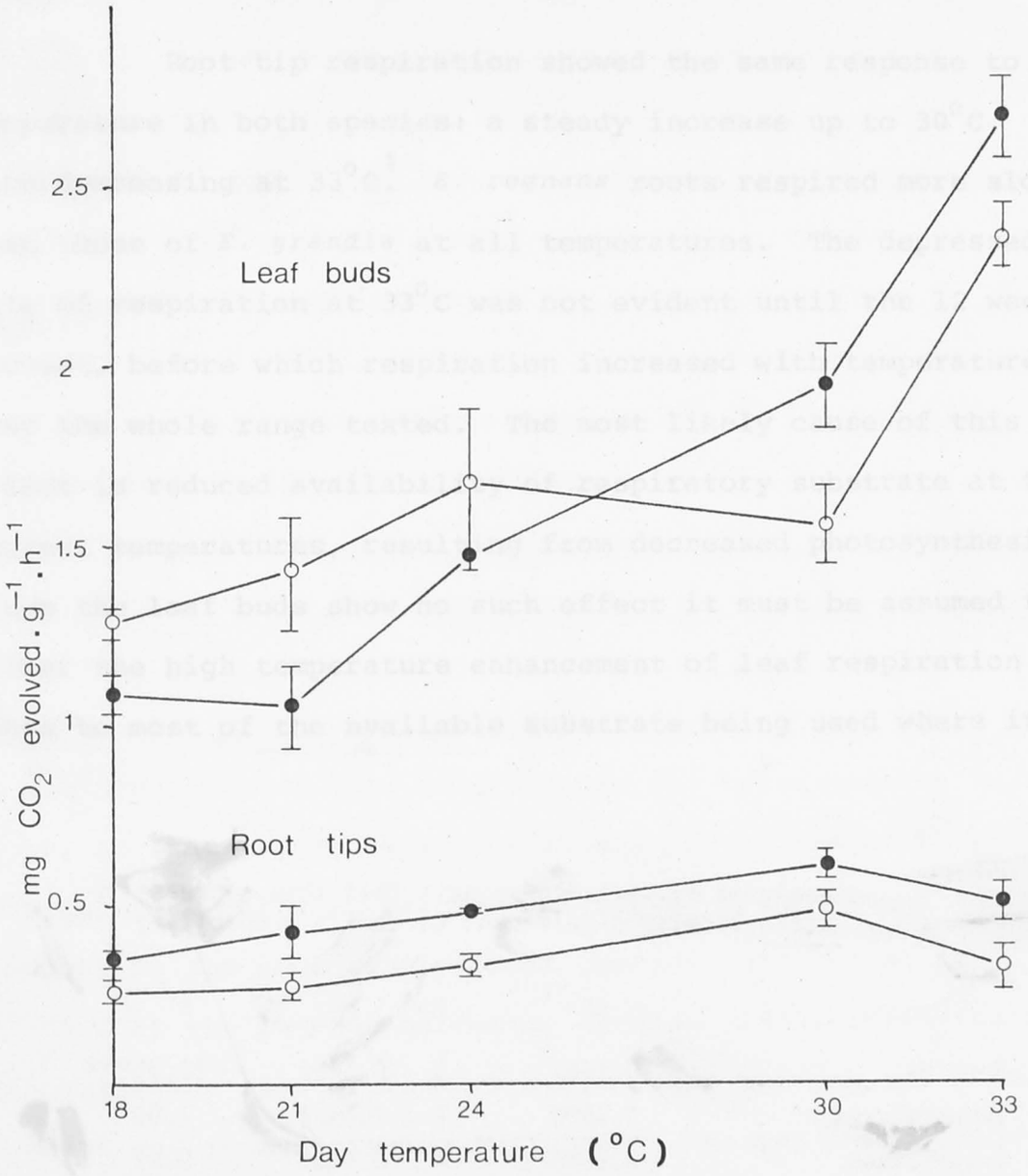


Figure 3-3. Dark respiration rates of leaf buds and root tips from seedlings of *E. regnans* (○) and *E. grandis* (●) at the 16 week harvest. Each point is the mean of 3 samples, ± standard error.

[1] Analysis of variance reveals that this decrease is statistically significant at the 1% probability level.

Source	SS	DF	MS	F
Species	0.1461	1	0.1461	24.35***
Temperatures	0.1895	4	0.0474	7.90***
Interaction	0.0064	4	0.0016	0.27
Error	0.1194	20	0.0060	
Total	0.4614	29		

A t-test of the difference of means from 30^{o} / 25^{o} and 33^{o} / 28^{o} C shows t= 2.931 with 20 degrees of freedom. **

synthesis) at high temperatures than those from a cooler climate. The reverse was true at low temperatures, clearly indicating the adaptation of each race to its normal environment.

Root tip respiration showed the same response to temperature in both species: a steady increase up to 30°C, then decreasing at 33°C.[1] *E. regnans* roots respired more slowly than those of *E. grandis* at all temperatures. The depressed rate of respiration at 33°C was not evident until the 12 week harvest, before which respiration increased with temperature over the whole range tested. The most likely cause of this effect is reduced availability of respiratory substrate at the highest temperatures, resulting from decreased photosynthesis. Since the leaf buds show no such effect it must be assumed that either the high temperature enhancement of leaf respiration leads to most of the available substrate being used where it is produced so that less is available for translocation to the roots, or that translocation is itself restricted at high temperatures. It will be seen in Chapter 4 that sugar concentrations in the root saps of both species were lowest at 33°/28°C at the 16 week harvest. Wardlaw (1972, 1974) found that while the rate of flow of assimilates through the phloem in wheat was insensitive to temperature changes over a wide range, both the rate of vein loading in the leaves and the rate of movement into the grain showed pronounced optima at 30°C. Pisek *et al.* (1973) report several other examples of the temperature dependence of transport processes.

[1] Analysis of variance indicates that this effect is statistically significant at the 0.1% probability level. Analysis has been performed on the untransformed data as Tukey's test and an examination of plot mean residuals revealed no evidence of non-additivity of treatment effects or heterogeneity of variances.

Source	SS	DF	MS	F
Species	0.0056	1	0.0056	3.73
Temperatures	0.2335	4	0.0584	38.93***
Interaction	0.1089	4	0.0272	18.13***
Error	0.0295	20	0.0015	
Total	0.3775	29		

A t-test of the difference of means for 30^{o} / 25^{o} C and 33^{o} / 28^{o}C shows t = 5.875 with 20 degrees of freedom.***

The absence of a depression in respiration rate at 33°C in *E. grandis* before the 12 week harvest or in *E. regnans* before the 16 week harvest suggests that the supply of photosynthate to the roots was not limiting in the early stages of growth; it may be relevant that the fraction of total dry weight in the leaves of the 33°/28°C seedlings did not fall below that of the 30°/25°C seedlings until the 12 week harvest in *E. grandis* and the 16 week harvest in *E. regnans*. At the 20 week harvest, respiration of *E. regnans* root tips at 30°C was less than at 24°C, which may represent a part of the same continuing decline in optimum temperature in this species as described in the previous Chapter.

The variation in respiratory quotient (RQ) of leaf buds and root tips with temperature at the 16 week harvest is shown in Figure 3-4. At the lower temperature RQ tended to fluctuate considerably between harvests, so that the low values shown by *E. grandis* at 18°/13°C cannot be taken as constant or representative over the whole period of growth. At higher temperatures however, there was very little change in RQ with time. Little difference between species is apparent, but there does appear to be an effect of high temperature on RQ of the root tips.[1] The decrease shown by both species at 33°C here, but not in the leaf buds, could also be seen as the result of decreased translocation of photosynthate to the roots leading to increasing respiration of protein or fat. Again, data to be presented in Chapter 4 indicate that protein concentrations in root saps of both species were lowest at 33°/28°C at the 16 week harvest.

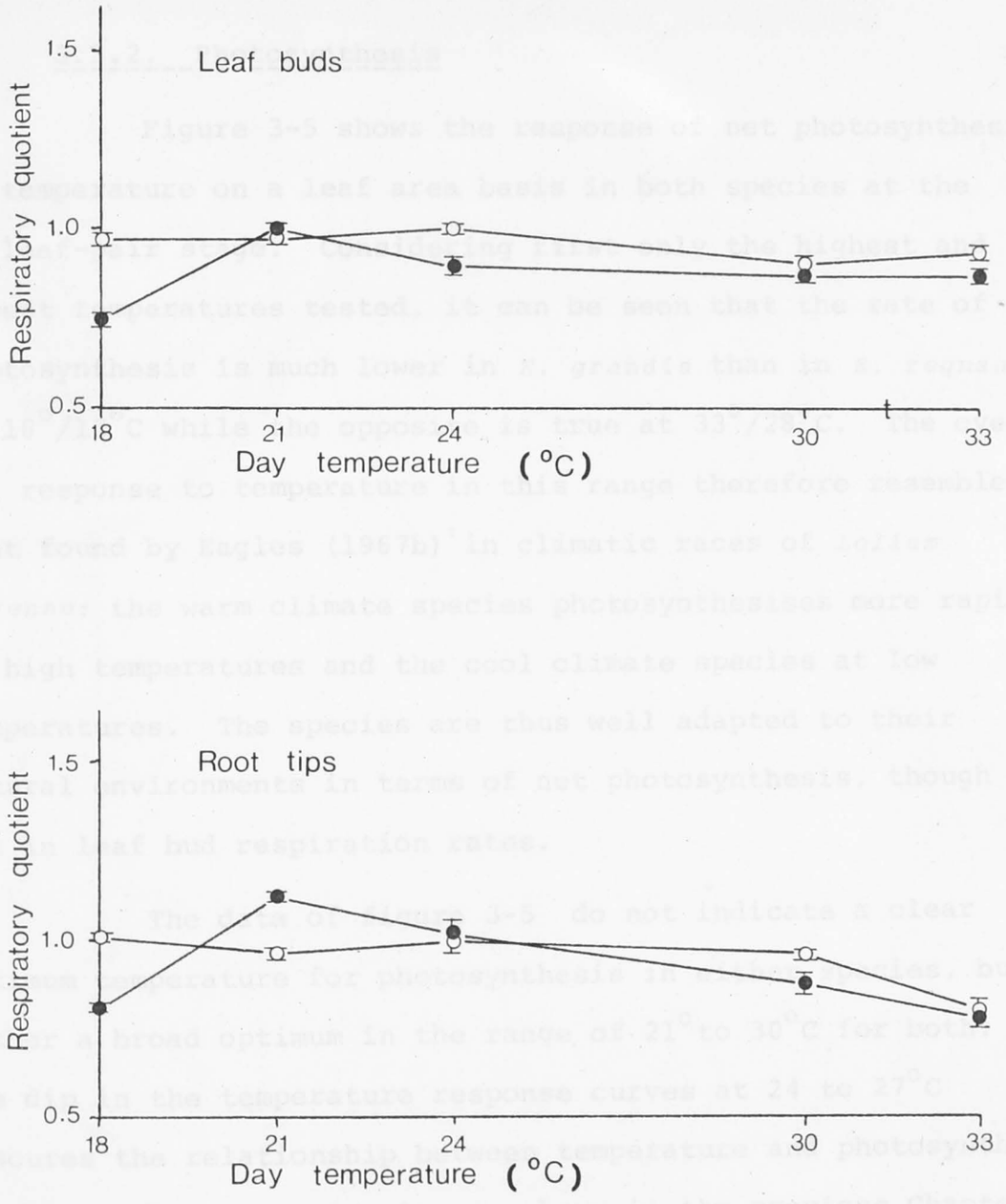


Figure 3-4. Respiratory quotients of leaf buds and root tips of *E. regnans* (○) and *E. grandis* (●) at the 16 week harvest. Each point is the mean of 3 samples, ± standard error.

3.3.2. Photosynthesis

Figure 3-5 shows the response of net photosynthesis to temperature on a leaf area basis in both species at the 15-leaf-pair stage. Considering first only the highest and lowest temperatures tested, it can be seen that the rate of photosynthesis is much lower in *E. grandis* than in *E. regnans* at $18^{o}/13^{o}C$ while the opposite is true at $33^{o}/28^{o}C$. The overall response to temperature in this range therefore resembles that found by Eagles (1967b) in climatic races of *Lolium perenne*: the warm climate species photosynthesises more rapidly at high temperatures and the cool climate species at low temperatures. The species are thus well adapted to their natural environments in terms of net photosynthesis, though not in leaf bud respiration rates.

The data of figure 3-5 do not indicate a clear optimum temperature for photosynthesis in either species, but rather a broad optimum in the range of 21^{o} to $30^{o}C$ for both: the dip in the temperature response curves at 24 to $27^{o}C$ obscures the relationship between temperature and photosynthesis in this range. However, it was shown in the previous Chapter (Figure 2-9) that the specific leaf area of the seedlings varied considerably between temperature regimes, particularly in *E. grandis*. As a result, when the photosynthesis measurements are expressed on a leaf fresh weight basis a clear optimum temperature of $30^{o}C$ in *E. grandis* seedlings becomes apparent. There is little change in the response of *E. regnans*, a broad optimum remaining in the range $21\text{-}30^{o}C$ as in Figure 3-5, and the relation between the temperature responses of the two species is otherwise unchanged.

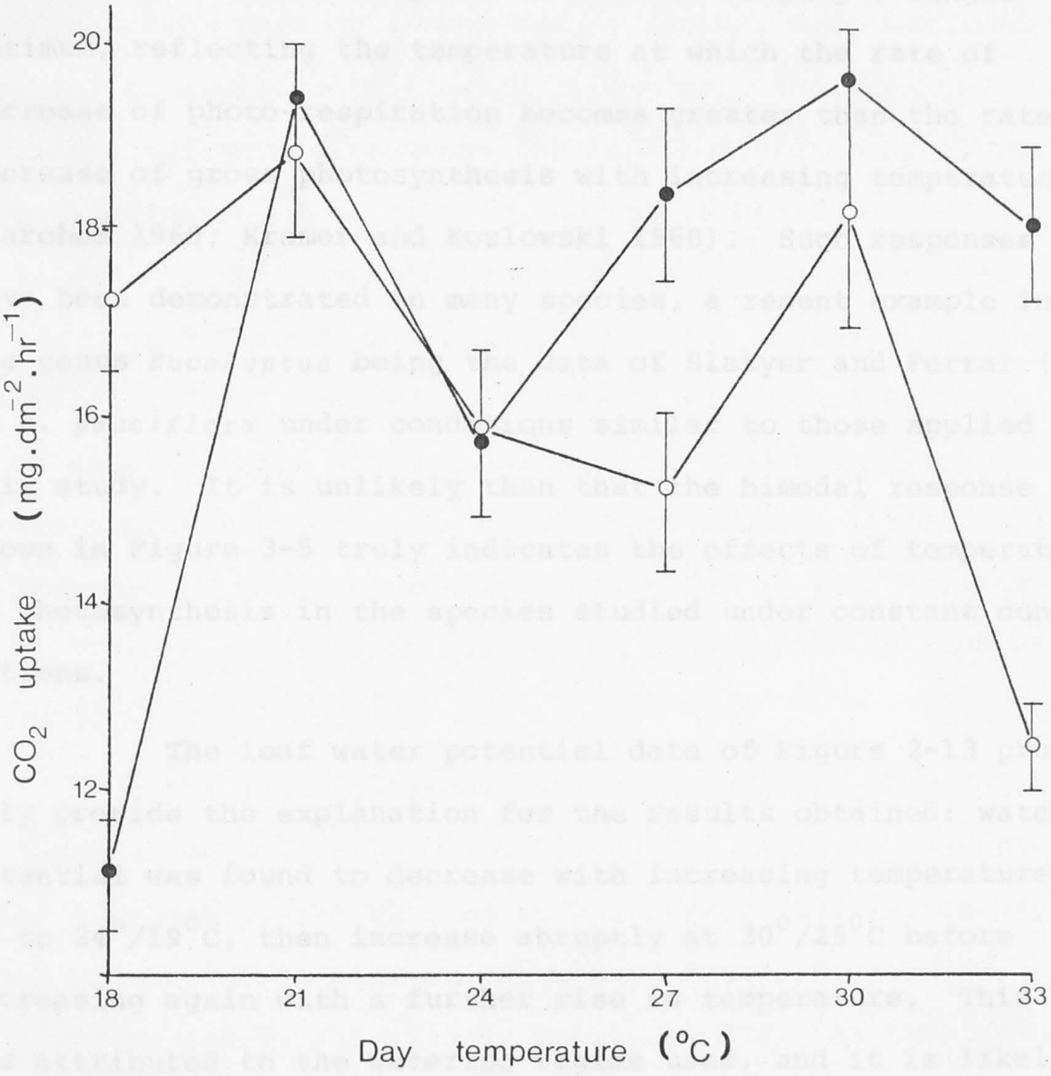


Figure 3-5. Net photosynthesis of *E. regnans* (○) and *E. grandis* (●) seedlings grown at a range of temperatures. Each point is the mean of 8 single attached leaves, ± standard error.

The dip in the photosynthetic temperature response curves at intermediate temperatures requires some comment. It is usual for such response curves to display a single optimum, reflecting the temperature at which the rate of increase of photo-respiration becomes greater than the rate of increase of gross photosynthesis with increasing temperature (Larcher 1969; Kramer and Kozlowski 1960). Such responses have been demonstrated in many species, a recent example in the genus *Eucalyptus* being the data of Slatyer and Ferrar (1977) on *E. pauciflora* under conditions similar to those applied in this study. It is unlikely then that the bimodal response shown in Figure 3-5 truly indicates the effects of temperature on photosynthesis in the species studied under constant conditions.

The leaf water potential data of Figure 2-13 probably provide the explanation for the results obtained: water potential was found to decrease with increasing temperature up to $24^{o}/19^{o}C$, then increase abruptly at $30^{o}/25^{o}C$ before decreasing again with a further rise in temperature. This was attributed to the watering regime used, and it is likely that the seedlings used for photosynthesis measurements were similarly affected so that seedlings grown at $24^{o}/19^{o}C$ and $27^{o}/22^{o}C$ had lower leaf water potentials than those from $21^{o}/16^{o}C$ or $30^{o}/25^{o}C$. As a result, the above estimate of optimum temperature for photosynthesis in *E. grandis* seedlings as $30^{o}C$ may be too high; 27^{o} would probably be better at the same water potential.

To obtain further evidence that differences in leaf water potential cause the dip in the photosynthetic response curves, the stomatal apertures of upper leaves of *E. regnans* seedlings from each of 5 temperature regimes were measured by means of an aspirated diffusion porometer. The results obtained indicated a tendency for leaf resistance to increase with temperature, from 0.70 $s.cm^{-1}$ at $18^{o}/13^{o}C$ to 1.43 $s.cm^{-1}$ at $33^{o}/28^{o}C$. The resistance at $30^{o}/25^{o}C$ was lower than at $24^{o}/19^{o}C$ or $33^{o}/28^{o}C$, corresponding to the increase in water potential at this temperature observed in other seedlings, but in no case were the differences between temperature regimes statistically significant. Hofstra and Hesketh (1969) found an increase in stomatal aperture (decrease in leaf resistance) of *E. regnans* leaves with increasing temperature in the range 18 to $33^{o}C$.

The peak rates of net photosynthesis recorded (up to about 23 $mg.dm^{-2}.hr^{-1}$ or 64 $ng.cm^{-2}.s^{-1}$) are of the same order as those listed by Slatyer and Ferrar (1977) from greenhouse and phytotron studies of *E. pauciflora*, *E. regnans*, *E. globulus* and *E. socialis* F. Muell. ex Miq. As they pointed out, these values are near the upper limit of the range of photosynthetic rates generally observed in trees, and are considerably higher than the rates attained by the same species under field conditions. Slatyer and Ferrar (1977) also found higher optimum temperatures in phytotron-grown material than those recorded by Slatyer and Morrow (1977) for trees in the field, a result which may help to explain the accelerated development and earlier failure of *E. regnans* at high temperatures under phytotron conditions compared with field observations from the Coffs Harbour species trial.

3.4. Discussion

Both photosynthesis and respiration are processes known to be directly affected by changes in temperature, and hence effects on them must be considered as possible causes of some of the growth differences between species and temperature regimes described in Chapter 2. The results obtained certainly explain some of the effects of temperature on growth of each species, but little evidence has been found to implicate differences in photosynthesis or respiration as major causes of the difference in the ability of *E. regnans* and *E. grandis* to tolerate high temperatures.

The data obtained on respiration rates indicate a difference in Q_{10} of leaf bud respiration in the two species, which leads to higher rates in *E. grandis* at high temperatures and in *E. regnans* at lower temperatures. This apparently poor climatic adaptation is reflected in the response of NAR to temperature (Figure 2-6), where it is probably responsible for NAR of *E. grandis* seedlings being less affected by low temperature than that of *E. regnans*, and for the lower NAR of the former species at $30^{\circ}/25^{\circ}C$ than at $24^{\circ}/19^{\circ}C$. The leaf bud respiration data do not help to explain the greater sensitivity of *E. regnans* to high temperature than *E. grandis*; the same applies to respiration of root tips, where the rates observed in the latter species were higher at all temperatures, and to respiratory quotients, which were generally similar in both species.

The root respiration rates and RQ values do however reveal a probable cause of the reduced growth rate, in part-

icular the root growth rate, of both species at $33^{o}/28^{o}C$: decreased respiration rates and lower RQ values suggest a restricted supply of photosynthate to the roots, which may result simply from the decreased photosynthesis and increased leaf respiration rates, or from a temperature-induced reduction in translocation. The first possibility is supported by the concomitant reduction in shoot growth observed, but the second remains attractive as it could result in restricted transport of mineral nutrients and root-synthesised hormones to the shoot, which could help to explain some of the morphological effects of supra-optimal temperature. In addition, the seedlings of *E. regnans* grown at $33^{o}/28^{o}C$ which died during the course of the growth study always died suddenly, with all leaves turning brown in a short time; this is distinct from the gradual decline of surviving trees at $33^{o}/28^{o}C$ and lower temperatures, in which browning of leaves was usually progressive from older to younger leaves and was accompanied by abscission. The sudden death of seedlings suggests failure of the root system rather than the result of gradual degrade of the shoot. Again, this could be due to root death from starvation which might or might not result from restricted transport from the leaves. The dependence of *E. regnans* seedlings on current photosynthate for root growth and seedling health has been shown by Wilson and Bachelard (1975).

The response of net photosynthesis to temperature in each species reveals a further cause of reduced growth rate in both species at high and low temperatures, but more importantly a real difference between species: photosynthesis in *E. grandis* is more sensitive to low temperature than in

E. regnans, and the reverse is true at high temperatures. This effect, which may derive from differences in the thermal stability of enzymes in the two species, is certainly a cause of the difference in optimum (and maximum) temperatures for growth. As the effect of seedling age on photosynthesis was not studied, the decreasing optimum temperature for growth of *E. regnans* cannot be ascribed to a similar change in the photosynthetic optimum; indications are that in phytotron-grown seedlings of this species peak photosynthesis occurs over a fairly broad range of temperatures, so if such a shift occurs it might be difficult to detect.

CHAPTER 4

EFFECTS OF TEMPERATURE ON ROOT SAP CONSTITUENTS OF *E. REGNANS* AND *E. GRANDIS* SEEDLINGS

4.1. Introduction

Among the ways in which a plant may react to a high (or low) temperature stress are changes in reaction rates and equilibria, enzyme activity, membrane permeability and translocation; such effects have been demonstrated by many workers in a wide range of plant species. It is reasonable to expect these effects to be reflected in the concentrations of the major translocated metabolites in the phloem and xylem sap, and such variations in concentration of sap components with temperature have been demonstrated (e.g. Waldron 1976).

Wilson and Bachelard (1975) reported qualitative and quantitative changes in sugars and amino acids in the root sap of *E. regnans* seedlings following decapitation, associated with the appearance of growth-inhibiting compounds and development of a brown colour, and leading to the death of the seedlings. Root saps of *E. viminalis* Labill. seedlings remained colourless and unchanged following the same treatment, and the seedlings survived by producing a new shoot system from epicormic buds. While apparently unrelated to the effects of high temperature on eucalypts under study here, these results provide another example of the failure of *E. regnans* seedlings to survive a stress (in this case removal of photosynthetic tissues) which some other species of *Eucalyptus* are able to overcome as a result of physiological adaptations.

As a difference in the sensitivity of *E. regnans* and *E. grandis* seedlings to a high temperature stress has been clearly demonstrated, it is of interest to determine the extent to which concentrations of root sap constituents of these two species vary with temperature. In particular, it is important to compare the effects of temperature between species, as a difference in sap concentrations of the kind found by Wilson and Bachelard (1975) might point toward one of the basic mechanisms leading to the greater sensitivity of *E. regnans* to high temperature stress; large increases in amino acid and sugar concentrations in this species might even indicate a common basis in seedling death following defoliation and that resulting from exposure to supra-optimal temperatures.

Root saps were therefore collected from seedlings of *E. grandis* and *E. regnans* and assayed for amino acids, sugars and soluble proteins. From the results obtained the effects of temperature on the concentrations of these major metabolites in each species can be compared at a range of seedling ages.

4.2. Materials and methods

Seedlings of *E. regnans* and *E. grandis* were grown in phytotron glasshouses as described in Chapter 2. At the time of each harvest of seedlings for measurement of leaf areas, dry weights, etc. (8, 10, 12, 16 and 20 weeks after transfer to different temperature regimes) root saps were extracted from an extra four or five seedlings grown at each temperature. Seedlings of *E. regnans* allowed to grow for 30 weeks at $18^{\circ}/13^{\circ}C$, $21^{\circ}/16^{\circ}C$, $24^{\circ}/19^{\circ}C$ and $30^{\circ}/25^{\circ}C$ were also used for this purpose.

Soon after removal from the phytotron, the stem of each seedling was severed approximately 5 cm above the stem - root junction. Potting medium was shaken from the roots and the bark removed from the base of the stem. The root system was then placed in a pressure bomb (Scholander *et al.* 1965) and sufficent pressure applied to maintain a flow of sap from the cut stem. The pressure required was usually 10^6 Pa or less, and flow continued for up to 30 minutes, yielding from less than 1 ml of sap in the smallest seedlings to as much as 25 ml from the largest. The sap from each seedling was collected into a separate vial and stored at $-20^{\circ}C$ until assayed.

Total sugar concentrations in the saps were determined by the phenol-sulphuric acid assay described by Bell (1955). Aliquots of sap (usually 0.1 ml) were made to 2 ml with distilled water and 0.14 ml of 80% aqueous phenol added, followed by 5 ml of concentrated H_2SO_4. The absorbance of the solution was measured at a wavelength of 490 nm against a blank containing no sap. The concentration of sugars in terms of glucose equivalent weight was determined by comparison of the absorbance with a calibration curve for standard concentrations of glucose. All assays were performed in duplicate.

Soluble proteins were assayed by the method of Lowry *et al.* (1951). To 0.3 ml aliquots of sap made to 1 ml with distilled water was added 5 ml of freshly prepared alkaline copper solution, followed after 10 minutes by 0.5 ml of 50% aqueous Folin reagent. The solutions were mixed immediately and colour development was allowed to proceed in darkness for 1 hour. The absorbance of each solution was then measured at a wavelength of 550 nm, and protein concentrations determined

by comparison with a calibration curve prepared by assay of standard concentrations of bovine serum albumin. All assays were again performed in duplicate.

Amino acids in the root saps were assayed by the ninhydrin colorimetric method of Rosen (1957). 0.1 ml aliquots of sap were brought to 1 ml with distilled water and 0.5 ml of cyanide-acetate buffer added, followed by 0.5 ml of 3% ninhydrin in 2-methoxyethanol. The solutions were heated in a water bath at 100°C for 15 minutes, then diluted with 5 ml of 50% aqueous isopropanol and mixed thoroughly. After cooling, the absorbance of each solution was measured at a wavelength of 570 nm and the concentration of amino acids determined by reference to a calibration curve prepared by assay of standard concentrations of leucine. As usual, all assays were performed in duplicate.

4.3. Results

The results of all assays are listed in Tables 4-1 to 4-3. Root saps of neither *E. regnans* nor *E. grandis* showed any tendency toward discolouration at high temperatures; in fact, the reverse was true. Saps from the lower temperatures tested were strongly yellow coloured, the intensity of the colour diminishing with increasing temperature such that saps from 33°/28°C were almost colourless. There was little difference between the two species in this respect, *E. grandis* saps being if anything slightly darker.

As discussed in more detail below, the similarity between species extends to the concentrations of sugars, proteins and amino acids determined, except perhaps in young *E. grandis*

seedlings at the lowest temperatures investigated. The standard errors shown in Tables 4-1 to 4-3 reveal that considerable variation was often present among saps of seedlings from the same harvest and temperature regime, in both species. While this might be expected to obscure the effects of temperature on concentration of the root sap components, there are in fact clearly identifiable trends in all those assayed (Figures 4-1 to 4-3); the variations in concentration with seedling age are more erratic and may indicate the existence of uncontrolled factors which influenced the root sap composition of the seedlings.

4.3.1. Sugars

Figure 4-1 shows the variation in total sugar concentration in root saps of *E. regnans* and *E. grandis* seedlings at the 8 and 16 week harvests. In general, concentration tends to decrease with increasing temperature; the major exception to this trend is seen at $33^{\circ}/28^{\circ}C$ in 8 week material of both species. However, by the 12 week stage (Table 4-1) sugar concentrations at $30^{\circ}/25^{\circ}C$ and $33^{\circ}/28^{\circ}C$ are nearly equal, and by 16 weeks concentrations at $33^{\circ}/28^{\circ}C$ have fallen behind those at $30^{\circ}/25^{\circ}C$.

The species differ most at the lower temperatures ($18^{\circ}/13^{\circ}C$ and $21^{\circ}/16^{\circ}C$), where the sugar concentrations in *E. grandis* sap was markedly higher, at least at the 8 week harvest. Although this difference appears to persist in the 16 week material, Table 4-1 indicates that it is a transient effect, with little or no difference between species at the 12 or 20 week harvests. The greatest effects of temperature

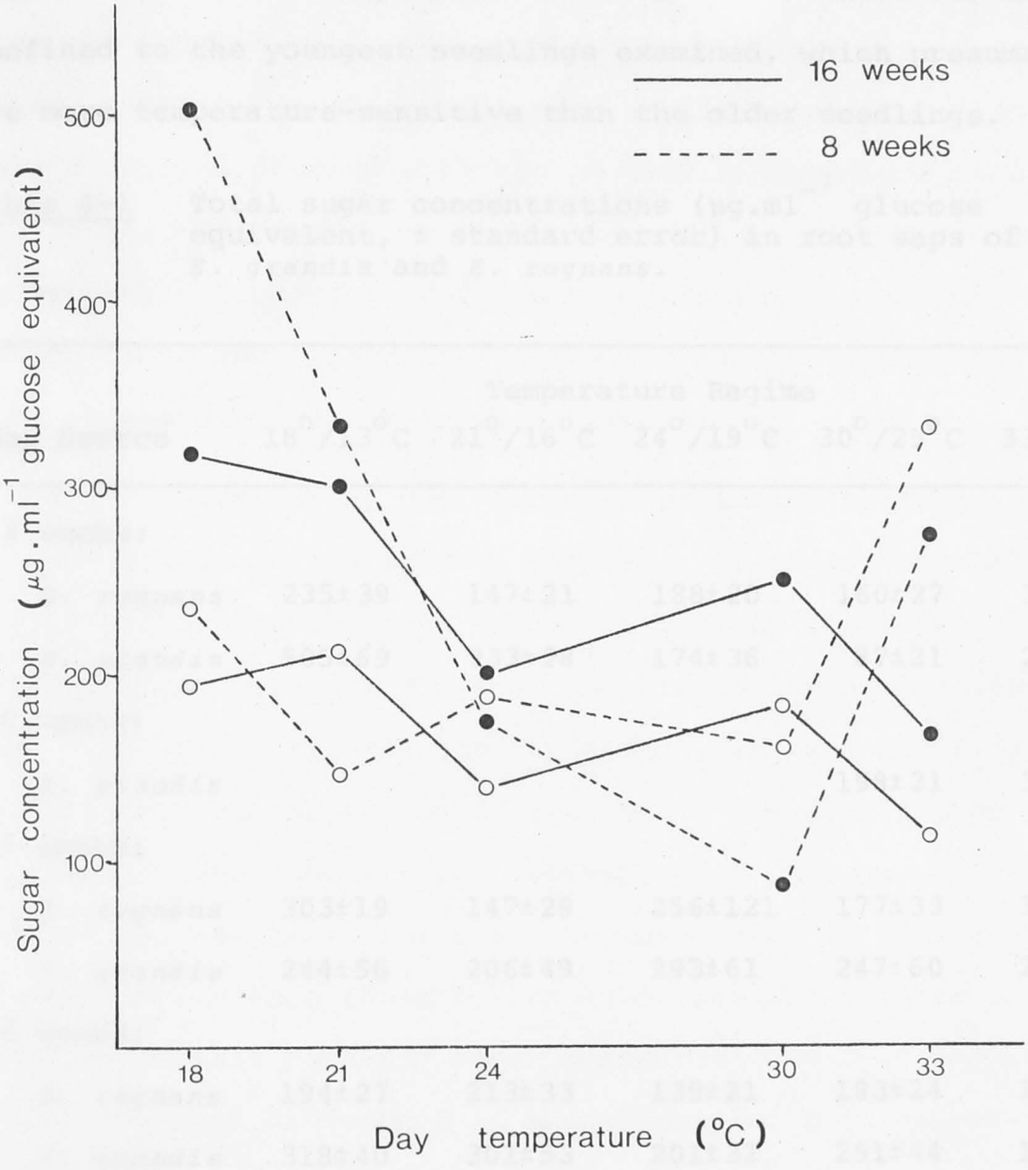


Figure 4-1. Variation in root sap sugar concentrations in *E. regnans* (○) and *E. grandis* (●) with temperature at the 8 and 16 week harvests.

on sugar concentration (increases at high temperature in both species and at low temperature in *E. grandis*) therefore are confined to the youngest seedlings examined, which presumably are more temperature-sensitive than the older seedlings.

Table 4-1 Total sugar concentrations ($\mu g.ml^{-1}$ glucose equivalent, ± standard error) in root saps of *E. grandis* and *E. regnans*.

Sap Source	Temperature Regime 18°/13°C	21°/16°C	24°/19°C	30°/25°C	33°/28°C
8 weeks:					
E. regnans	235±39	147±21	188±20	160±27	332±27
E. grandis	505±69	333±28	174±36	87±21	275±49
10 weeks:					
E. grandis				198±21	332±47
12 weeks:					
E. regnans	303±19	147±29	256±121	177±33	187±37
E. grandis	244±56	206±49	293±61	247±60	221±42
16 weeks:					
E. regnans	194±27	213±33	139±21	183±24	113*
E. grandis	318±40	301±53	201±32	251±44	168±37
20 weeks:					
E. regnans	275±58	198±49	317±49	185±26	
E. grandis	329±59	286±58	568±67		
30 weeks:					
E. regnans	104±8	250±22	145±58	189±6	

* One sample only

No clear effect of seedling age can be inferred from the data of Table 4-1, perhaps partly as a result of the large variation between seedlings. While sugar concentrations show a general decline with age at some temperatures, they tend to increase at others, or fluctuate considerably from one harvest to another. Uncontrolled factors or irregular practices such as repotting and glasshouse fumigation may be responsible for some of the differences between harvests.

Considering the amount of variation observed both between seedlings and between harvests, there is remarkably little difference between species except as mentioned at the lowest temperatures at 8 weeks; the fluctuations that occur with time are mostly the same in both *E. regnans* and *E. grandis*, and although the sugar concentrations in *E. grandis* saps are generally higher than in the corresponding *E. regnans* saps, most of the estimates for each species differ by less than one standard error from those of the other species. There is thus no evidence that root sap sugar concentrations bear any relation to the observed difference in the effects of high temperature on the growth of the two species.

4.3.2. Proteins

Protein concentrations also tend to decrease with increasing temperature (Figure 4-2). Again, saps from the 8 week harvest appear to be exceptional in that concentrations increase at the highest as well as lowest temperatures; as in the sugar concentrations, this effect has disappeared by 12 weeks (Table 4-2).

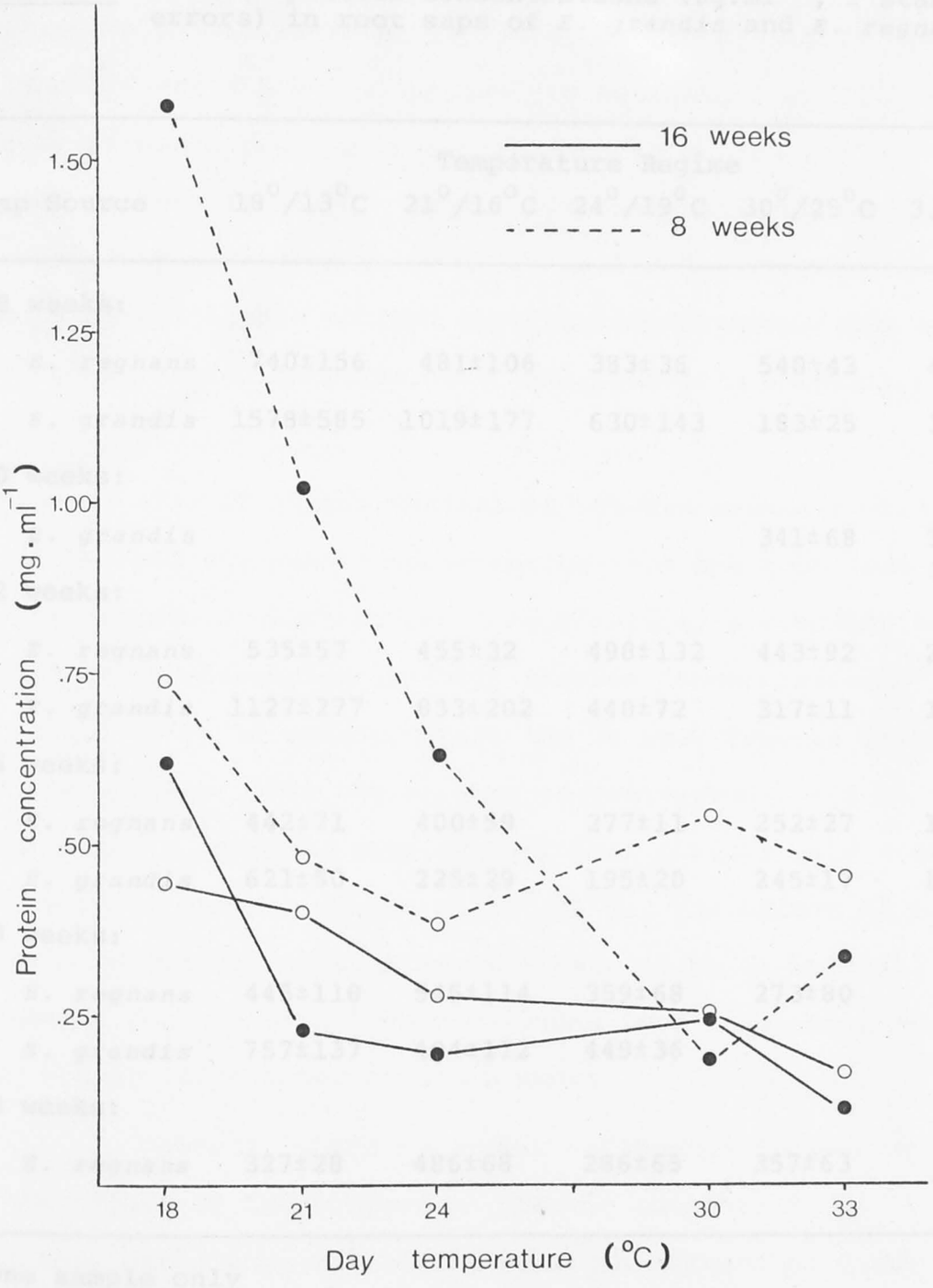


Figure 4-2. Variation in root sap protein concentrations in *E. grandis* (●) and *E. regnans* (○) with temperature at the 8 and 16 week harvests.

Table 4-2 Soluble protein concentrations ($\mu g.ml^{-1}$, ± standard errors) in root saps of *E. grandis* and *E. regnans*.

Sap Source	Temperature Regime 18°/13°C	21°/16°C	24°/19°C	30°/25°C	33°/28°C
8 weeks:					
E. regnans	740±156	481±106	383±36	540±43	451±29
E. grandis	1578±585	1019±177	630±143	183±25	335±89
10 weeks:					
E. grandis				341±68	378±73
12 weeks:					
E. regnans	535±57	455±32	498±132	443±92	292±42
E. grandis	1127±277	833±202	440±72	317±11	173±11
16 weeks:					
E. regnans	442±71	400±59	277±11	252±27	163*
E. grandis	621±50	225±29	195±20	245±17	108±13
20 weeks:					
E. regnans	445±110	545±114	359±68	273±80	
E. grandis	757±137	404±112	449±36		
30 weeks:					
E. regnans	327±28	486±68	286±65	357±63	

* One sample only

The major difference between species also mirrors that discussed above with reference to sugar concentrations: root saps of *E. grandis* seedlings at the 8 week harvest at lower

temperatures contain considerably more protein than those of *E. regnans*. This is so at 18°/13°C, 21°/16°C, and 24°/19°C; at the 12 week stage the species are equivalent at 24°/19°C, and by 16 weeks protein concentrations in *E. grandis* are higher only at 18°/13°C. This is probably an expression of the greater sensitivity of *E. grandis* to "low" temperatures as a result of its higher optimum growing temperature. This sensitivity appears to decrease with time, possibly a conditioning effect.

The irregular variations between harvests which characterised the sugar concentration data are much less marked in the protein concentrations. As a result, a seedling age effect can be discerned: in both species concentrations decreased up to 16 weeks. While the 20 week results mostly show an increase over the 16 week concentrations, the 30 week results for *E. regnans* saps suggest that this is only a transient effect, except perhaps at 30°/25°C. The effect of age on protein concentration thus seems likely to be an initial decrease from the high levels of young seedlings, to a more or less steady value after about 16 weeks.

Overall, there is little difference between species in the effect of temperature on protein concentrations in the root sap, other than the low temperature effects on young *E. grandis* seedlings mentioned above. As with the sugar concentrations, the similarities between species are more notable than the differences, even at high temperature. Therefore there is no evidence for an effect of temperature stress on protein concentration in *E. regnans* root saps exceeding the effect in *E. grandis*. Like the sugar concentrations, protein

concentrations do not appear to be related to the large difference in effects of supra-optimal temperature on growth of *E. regnans* and *E. grandis* seedlings.

4.3.3. Amino acids

The variation in amino acid concentrations with temperature at the 8 and 16 week harvests is shown in Figure 4-3. Again, it can be seen that concentrations decrease with increasing temperature. There is little evidence of an increase in concentrations at high temperature as seen in the sugar and protein assays, even in the youngest material examined. There is, however, a tendency to level off at the high temperatures, the largest concentration differences occurring between $18^{o}/13^{o}C$ and $24^{o}/19^{o}C$.

The amino acid concentrations show even less difference between species than did the proteins and sugars: Table 4-3 shows that even at $18^{o}/13^{o}C$, concentrations in each species are within one standard error of those in the other species at all harvests except 20 weeks. At $33^{o}/28^{o}C$ there is some evidence of a species effect, concentrations in *E. grandis* falling to quite low levels after 12 weeks while those in *E. regnans* remain more or less stable.

As in the sugar concentration data, large fluctuations in amino acid concentration occur from one harvest to the next, particularly at the lower temperatures (Table 4-3). No clear effect of seedling age can be seen as a result. The similarity of these irregular variations in both species however serves to reinforce the conclusion that, like sugars and proteins, amino acid concentrations differ very little

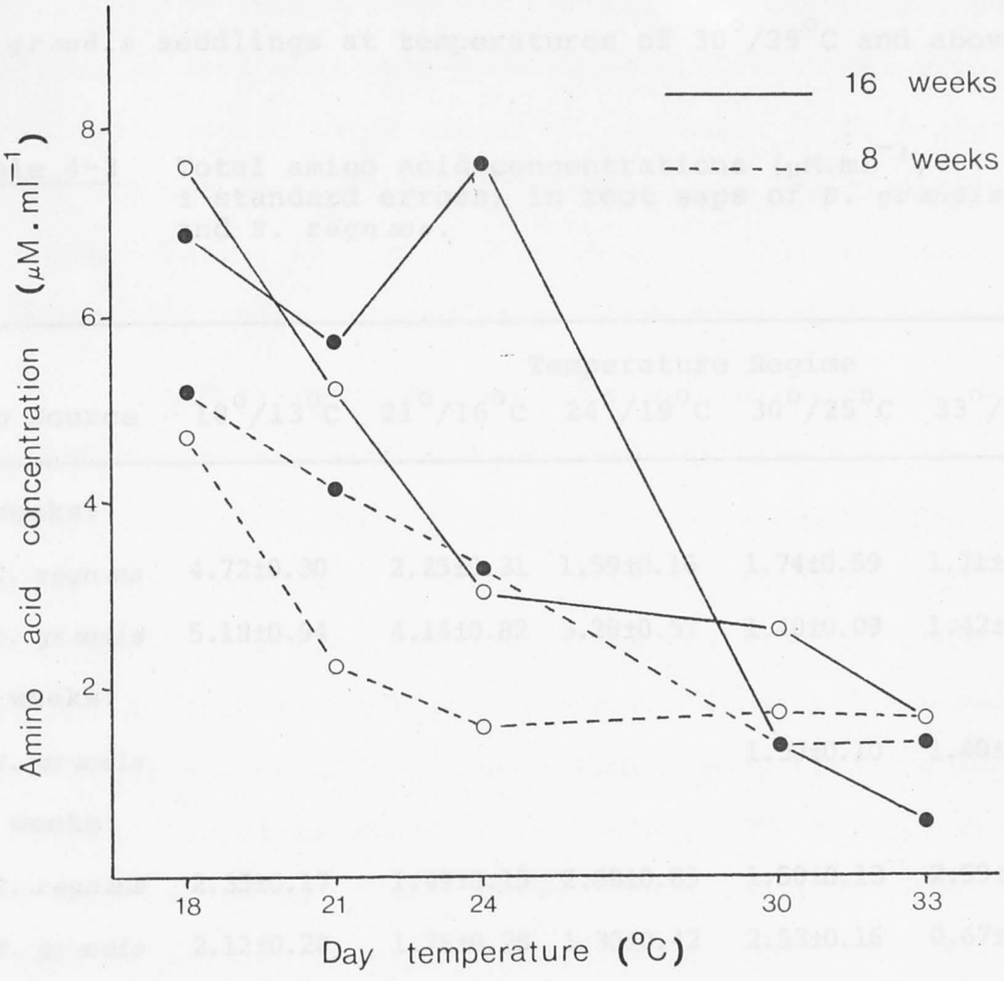


Figure 4-3. Variation in root sap amino acid concentrations in *E. grandis* (●) and *E. regnans* (○) seedlings with temperature at the 8 and 16 week harvests.

between species. These compounds therefore also appear to be unrelated to the differing behaviour of *E. regnans* and *E. grandis* seedlings at temperatures of $30^{\circ}/25^{\circ}C$ and above.

Table 4-3 Total amino acid concentrations ($\mu M.ml^{-1}$, ± standard errors) in root saps of *E. grandis* and *E. regnans*.

Sap Source	Temperature Regime $18^{\circ}/13^{\circ}C$	$21^{\circ}/16^{\circ}C$	$24^{\circ}/19^{\circ}C$	$30^{\circ}/25^{\circ}C$	$33^{\circ}/28^{\circ}C$
8 weeks:					
E. regnans	4.72±0.30	2.25±0.31	1.59±0.16	1.74±0.59	1.71±0.12
E. grandis	5.18±0.94	4.14±0.82	3.28±0.57	1.40±0.09	1.42±0.27
10 weeks:					
E. grandis				1.30±0.10	1.40±0.12
12 weeks:					
E. regnans	2.35±0.17	1.49±0.15	2.68±0.85	1.50±0.13	2.59±0.60
E. grandis	2.12±0.20	1.76±0.28	1.30±0.42	2.53±0.16	0.67±0.11
16 weeks:					
E. regnans	7.61±0.95	5.22±0.53	3.05±0.43	2.66±0.21	1.72*
E. grandis	6.87±0.54	5.73±0.99	7.66±0.74	1.41±0.19	0.59±0.18
20 weeks:					
E. regnans	3.09±0.27	4.23±0.66	4.13±0.97	2.48±0.58	
E. grandis	3.85±0.33	3.01±0.78	2.90±0.56		
30 weeks:					
E. regnans	1.89±0.06	2.74±0.52	2.02±0.38	2.63±0.42	

* One sample only

4.4. Discussion

The assays of sugar, protein and amino acid concentrations in root saps have demonstrated effects of growing temperature on all three groups of compounds in both species. As pointed out earlier, it is reasonable to expect such a phenomenon as the result of effects on a number of temperature-dependent physiological processes at the sub-cellular level.

A difference between species, in the extent to which concentrations vary with temperature, has also been located; unexpectedly, it is only *E. grandis* at the lower temperatures studied which displays unusually high concentrations of protein and sugars in the root sap. This species has been shown to have a higher optimum temperature for growth than *E. regnans*, but while seedlings at $18^{o}/13^{o}$C grew slowly they remained healthy and showed few other signs of low temperature stress, apart from some reddening of the leaves. Waldron (1976) found a similar effect in the xylem sap of sugar cane, where amino nitrogen levels were much higher at 18^{o}C than at 25^{o}C or 30^{o}C.

The species difference which might have been expected at $30^{o}/25^{o}$C, where *E. regnans* seedlings display stress symptoms but *E. grandis* seedlings thrive, was not found. Even at $33^{o}/28^{o}$C neither species shows any change in concentrations apart from the general downward trend with increasing temperature; there are exceptions in the youngest material assayed, and possibly in a lower concentration of amino acids in *E. grandis* sap from older material. The possibility of low sugar and protein concentrations at $33^{o}/28^{o}$C resulting from reduced supply of photosynthate to the roots has been referred to in Chapter 3. Certainly no large increase in sugar and

amino acid concentrations (of the order of 10- to 20- fold) as found in decapitated seedlings of *E. regnans* by Wilson and Bachelard (1975) occurs as a result of high temperature stress, even in the most severely affected plants. The absolute concentrations of sugars and amino acids in the saps examined here were of the same magnitude as those found by Wilson and Bachelard (1975) in intact *E. regnans* seedlings.

Apart from the effects of low temperature on *E. grandis*, both species are very similar, not only in the effects of temperature on root sap concentrations but even in the absolute levels of sugars, proteins and amino acids in the sap. The absence of a species difference, particularly at high temperatures, is surprising in view of the large difference in response of the two species to elevated temperatures. However, it provides an indication that the basis of the difference in temperature optima is not a phenomenon which would affect concentrations of the root sap components investigated, nor does it act through effects on their concentrations.

CHAPTER 5

THE INVOLVEMENT OF PLANT GROWTH SUBSTANCES IN HIGH TEMPERATURE EFFECTS

5.1. Introduction

The results of experiments described in Chapters 2 to 4 have demonstrated effects of temperature on a large number of characteristics of *E. regnans* and *E. grandis* seedlings. For the purposes of this study it is those effects which occur at temperatures above the optimum for growth which are of particular interest: in the seedlings studied these included, in addition to reduced growth rate, effects on leaf size, shape and colour; increased leaf and branch death and abscission, with replacement by epicormic shoots; limpness, plasticity and disordered growth of stems and branches, with excessive branch development at the expense of stem growth; shorter internodes and thinner stems; decreased branch angle; and eventual mortality of seedlings. Although *E. grandis* maintained satisfactory growth at the supra-optimal temperature regime of $33^{\circ}/28^{\circ}$C and was by no means affected as severely as *E. regnans*, most of these symptoms were shown by both species: the species do not appear to differ in their actual responses to supra-optimal temperature, but in the temperatures above which these responses become evident and in the rate of increase in severity of the effects with increasing temperature.

Similarly, respiration rates of leaf buds and root tips follow the same trends with temperature in both species,

a decrease in root respiration and respiratory quotient at $33^{o}/28^{o}$C suggesting the possibility of a root starvation effect; in older seedlings of *E. regnans* the same effect may occur at $30^{o}/25^{o}$C. The optimum temperature for photosynthesis in *E. grandis* leaves appears to be higher than in *E. regnans*, and the decline at supra-optimal temperatures steeper in the latter species. The concentrations of sugars, proteins and amino acids in the root saps of the two species are very similar, generally decreasing with increasing temperature.

To fulfil its objective of examining the physiological basis of the difference in the response of *E. regnans* and *E. grandis* to elevated temperatures, this project must seek to explain the differences between the species which enable one to grow vigorously at a temperature which is lethal to the other, rather than examining further the effects of supra-optimal temperature on physiological processes as the results so far suggest that these will not differ greatly between species. The questions to which answers must be sought are (i) what chemical difference exists between species to bring about the difference in optima? and (ii) what is the basis of the decrease in the optimum temperature for *E. regnans* (but not *E.grandis*) with seedling age?

The study of root sap constituents described in the previous chapter might have been expected to yield some clue to the nature of these species differences, but did not, as both species showed the same response to high temperatures and seedling age. The possibility of differences in the thermal stability of enzymes has already been mentioned in

connection with the difference in photosynthetic optima, and will be considered further in Chapter 11. A promising first line of investigation, given their widespread involvement in the regulation of physiological processes and their susceptibility to temperature effects, is to examine the variation in growth substances between species and temperature regimes. Qualitative or quantitative differences in these substances could readily explain most or all of the effects of high temperature observed, and the species difference could then be seen as a difference in the thermal stability of the growth substances present or of the systems which synthesise them.

5.2. Growth substances and mechanisms of heat tolerance

Levitt (1972) has extensively reviewed the effects of high temperature stress on plants and the mechanisms by which resistant species are able to avoid or tolerate them. He classified stress injuries first as either primary or secondary, depending on whether the injury resulted from the stress directly or from another factor induced by it. In the case of high temperature stress, the only important secondary stress injury is a heat-induced drought injury resulting from increased transpiration at high temperatures. Under phytotron conditions such a stress is unlikely to be important, and the effects of high temperature on eucalypt seedlings must be considered as primary heat injuries. Levitt (1972) further classified these as direct and indirect injuries, on the basis of the initial reversibility of the effects of an applied stress.

Primary direct heat injury is induced by a brief exposure to high temperatures, in the range of a few seconds to a few minutes; the temperature required to cause such effects is generally greater than 45°C. The injury is considered to result from loss of membrane semipermeability following the denaturation and aggregation of membrane proteins, possibly combined with lipid liquefaction and nucleic acid denaturation. At higher temperatures (greater than about 60°C) further injury from chemical decomposition becomes important. The high temperature and brief exposure time associated with this type of injury suggest that it is not involved in the effects on eucalypt growth and development under study here.

Primary indirect, or metabolic, heat injury is observed in different species at temperatures in the range of 15 to 45°C, and occurs gradually: plants may recover quickly from a brief exposure to such a stress, while longer exposures require a longer recovery time. In the early stages the effects are usually reversible (elastic strain), e.g. an increase in respiration rate or decrease in photosynthesis, but if the stress is prolonged irreversible effects will result (indirect plastic strain), e.g. death of leaves or other organs. The effects of supra-optimal temperature on *E. regnans* and *E. grandis* seedlings described in the preceding chapters clearly fit into this category.

Levitt (1972) described four mechanisms of metabolic heat injury: starvation, toxicity, biochemical lesions and protein breakdown. Growth substances could be involved either in these effects or in the means by which heat tolerant plants avoid them. The possible involvement of growth substance

[1]Pisek *et al.* (1968)

changes in each mechanism, either as causes or effects of other metabolic injury, and the possibility of species differences in the thermostability of the growth substances present conferring greater heat tolerance on *E. grandis* than *E.regnans*, are discussed in the following sections.

5.2.1. Starvation injury

At temperatures above the compensation point at which the rate of respiration of photosynthate equals its rate of accumulation by photosynthesis[1], the reserve foods of a plant become depleted and the plant may eventually die from starvation. At moderate temperatures this is usually the slowest kind of heat injury (Levitt 1972) and as such it may be of importance in the delayed failure of *E. regnans* at $30^{\circ}/25^{\circ}$C and lower temperatures. It has been seen already that a starvation effect in the roots and other non-photosynthetic organs may occur at temperatures below the photosynthetic compensation point if transport processes are affected by temperature.

Plants may avoid this kind of injury and thus increase their tolerance of high temperature stress by means of a high compensation point, resulting from a relatively slow increase in respiration or decrease in photosynthesis with increasing temperature. The results of leaf respiration and photosynthesis measurements discussed in Chapter 3 show that photosynthesis of *E. regnans* seedlings falls off more rapidly above 30°C than that of *E. grandis*, and in spite of the slightly

more rapid increase in respiration with temperature in *E. grandis* leaf buds, this effect is probably responsible in part for the lower thermotolerance of *E. regnans*.

It is now recognised (Leopold and Kriedemann 1975) that photosynthesis, like most physiological processes, may be under hormonal control, probably through effects on leaf and mesophyll diffusive resistances. Although the effects of temperature on photosynthesis probably occur primarily by control of the activity of the enzymes concerned (Larcher 1969), hormonal regulation may also be involved. This raises the possibility of growth substances affecting the compensation point, so that different growth substances or differences in their thermostability between species might be responsible for a difference in heat tolerance through this mechanism.

5.2.2. Toxicity injury

This type of injury becomes evident more rapidly than starvation effects, resulting from metabolic disturbances at high temperatures. Changes in reaction rates and in the position of equilibria, decreased availability of gases due to reduction in solubility with increasing temperature and related physico-chemical phenomena may exert profound and complex effects on all aspects of cell metabolism. One possible result is the production of toxic substances, or toxic quantities of metabolites usually present in much lower concentrations; an example is the accumulation of ammonia as a product of anaerobic respiration when aerobic respiration has been blocked, e.g. by reduced availability of oxygen.

[1]Petinov and Molotkovsky (1957)

Thermotolerant plants can avoid toxicity injury either by decreasing the production of toxic intermediates or by detoxifying those produced, e.g. the synthesis of organic acids to neutralise ammonia from anaerobic respiration and prevent its accumulation to toxic levels.[1] Toxicity injury might be expected to lead to effects on the growth substances present, depending on the reactions involved in the injury, or the concentrations of specific growth substances themselves could be increased to abnormal levels where they might either act as toxins, or disrupt the normal growth and development of the plant (e.g. the promotion of excessive branch growth).

5.2.3. Biochemical lesions

This type of injury results from metabolic disturbances similar to those leading to toxicity injury, and is in essence the opposite effect: rather than excessive quantities of undesirable compounds accumulating in the plant, inadequate quantities of essential substances are produced. In this case the effects of the stress can be overcome by providing an external supply of the key substances. Examples of this effect in higher plants are described by Ketellapper and Bonner (1961) and Langridge and Griffing (1959), who found lesions specific for vitamins, ribosides and sucrose. Langridge (1963) discussed the possible causes of lesions in some detail, and suggested that they could result from enzyme inactivation, effects on nucleic acids, rate imbalances, accelerated breakdown of metabolites and decreased availability of gases.

Species which avoid biochemical lesions may do so by achieving a higher rate of production of the compounds involved, if the cause of the lesion is accelerated breakdown at high temperatures. The existence of alternative metabolic pathways may also enable some species to by-pass the processes requiring the affected substance. Where the lesion results from enzyme inactivation, increased thermostability of the enzymes involved will confer resistance to heat injury of this type. It is conceivable that the same effects which result in biochemical lesions might lead to an acute deficiency of a specific growth substance at high temperatures, just as metabolic imbalances could cause excessive production of these substances. However, no instances of high temperature lesions for growth substances have been reported.

5.2.4. Proteolytic injury

Metabolic effects of high temperature may result in a loss of enzymes and other proteins from cells, either by an increased rate of destruction or decreased rate of synthesis. Such a decrease in protein synthesis could result from effects on nucleic acid metabolism, or from denaturation of protein-synthesising enzymes. Levitt (1972) suggested that the enzymes involved might be denatured at temperatures lower than those necessary for the denaturation of membrane proteins associated with direct heat injury, as a result of their lower hydrophobicity.

Plants may avoid proteolytic injury through an ability to repair or replace damaged protein; for example thermotolerant species may contain larger amounts of nucleic acids than related heat-sensitive species. Alternatively, thermotolerance may be conferred by the presence of protective substances; an illustration of this effect is the prevention of heat-induced yellowing of leaves by spraying with kinetin before or after heating (Engelbrecht and Mothes 1960). Finally, where denaturation of enzymes is involved, Levitt (1969, 1972) considered that enzyme thermostability (and hence avoidance of proteolytic injury from this cause) is achieved by a relatively high ratio of hydrophobic to hydrophilic groups in the protein. A reduced number of sulphhydryl and other groups capable of forming covalent intermolecular bonds may confer further heat-hardiness, but this effect is probably more important at the higher temperatures capable of causing direct injury by denaturing membrane proteins.

Once again, a role for growth substances either in causing or preventing proteolytic injury can readily be postulated. There is abundant experimental evidence implicating all the major growth substances (auxins, gibberellins, cytokinins, inhibitors) in the regulation of nucleic acid-directed protein synthesis (Leopold and Kriedemann 1975), and the delay of leaf senescence by cytokinin is believed to result from a retardation of protein breakdown; the above example of kinetin acting as a protective agent is an expression of such regulation. Thus, differences in the growth substances present in plant species may be responsible for differences in their tolerance of high temperatures. Alternatively,

effects of temperature on growth substances may lead to proteolytic injury through disturbance of the regulation of protein synthesis and breakdown.

5.3. The involvement of growth substances in temperature responses

The possibility of growth substances being involved in the major mechanisms of heat injury and thermotolerance in plants has now been established, and the likelihood of their association with the effects of high temperature observed on eucalypt seedlings can be considered. Auxins, gibberellins, cytokinins and inhibitors have all been implicated in a wide range of growth and morphological effects and physiological processes, so that it is not difficult to find examples of their involvement in almost all the effects displayed by *E. regnans* and *E. grandis* seedlings at supra-optimal temperatures. Much less is known of the effects of temperature on endogenous hormone levels, but the available data generally support the suggestion that effects of temperature on plant growth and development may result in part from effects on growth substance concentrations.

All the major types of growth substances have been suggested as regulators of stem elongation in at least some species; auxins and gibberellins appear to be of greatest importance generally. In eucalypts, Bachelard (1969a) demonstrated effects of gibberellic acid on internode elongation in *E. camaldulensis* Dehn., and Blake (1976) found a correlation between height growth and the concentration of an inhibitor in *E. obliqua* seedlings. A number of studies have indicated that cambial activity and diameter growth are promoted by the same

hormones which stimulate stem elongation, principally auxin (Kramer and Kozlowski 1960). Effects of auxin and gibberellin on cell walls (Audus 1972) could bring about the limpness and plasticity of stems and branches observed in *E. regnans* seedlings at high temperatures.

The excessive branch growth and decreased branching angle displayed by eucalypt seedlings at supra-optimal temperatures indicate a reduction in apical control (Brown *et al.* 1967) while the development of epicormic shoots reflects loss of apical dominance. Both of these phenomena are believed to be regulated by growth substances (Leopold and Kriedemann 1975). Blake (1973) studied effects of thermoperiod on apical dominance and apical control in *E. obliqua* seedlings, and concluded that these effects may be regulated by a balance between levels of gibberellin and cytokinin. In earlier work on apical dominance in relation to sprouting from *E. obliqua* lignotubers (Blake and Carrodus 1970; Blake 1972) it was suggested that both growth promoting and growth inhibiting substances in leaves are involved in the control of apical dominance. Field and Jackson (1974) also considered apical dominance to be regulated by a balance of hormones, and suggested that auxin, gibberellin and cytokinin levels were important. Bachelard (1969b) showed that the formation of epicormic shoots on stem segments of *E. polyanthemos* Schau. was inhibited by a variety of factors including auxin, gibberellic acid and kinetin, supporting the hypothesis that epicormic shoot formation is inhibited by factors which promote cambial activity.

Abscission also appears to be regulated by growth substances, the major substances involved being auxin as an inhibitor and ethylene as a promoter, although gibberellins, cytokinins and abscisic acid can all promote abscission under some circumstances (Leopold and Kriedemann 1975). The observed effects of high temperature on leaf morphology may also be mediated by growth substances; in particular, cytokinins are able to stimulate both leaf enlargement (Miller 1956) and chloroplast development (Stetler and Laetsch 1965).

There is thus abundant evidence that growth substances may regulate the morphological effects observed in eucalypt seedlings at supra-optimal temperatures. There is less direct evidence that growth substance concentrations do in fact vary with temperature. Hillman and Galston (1961) considered that auxin levels in plant tissues are not affected by temperature, although the rate of transport of auxin has been shown to increase with temperature up to an optimum, then decline (Gregory and Hancock 1955); this could bring about an effect of temperature on the available concentration of auxin at sites remote from the site of synthesis. The same can be said of other hormones which are translocated to remote parts of the plant: effects of temperature on the metabolic loading and unloading of substances into and out of the phloem stream lead to an optimum temperature for translocation also (Pisek *et al.* 1973). The low temperature inhibition of abscisic acid transport reported by Ingersoll and Smith (1971) may be a result of this general effect.

Itai and Benzioni (1974) found a reversible increase in abscisic acid and decrease in cytokinin concentrations to

result from brief exposure of leaves or roots to temperatures of 46°C to 49°C. A similar change in abscisic acid concentrations may be involved in the reversible increase in permeability of beet root cell membranes at 35 to 45°C described by Toprover and Glinka (1976), since Andres and Smith (1976) suggested an increase in membrane permeability as the cause of leakage of amino acids from *Lemna* cells treated with abscisic acid.

Other studies provide indirect evidence for effects of temperature on growth substances. Blake (1972) found that concentrations of growth promoting and inhibiting substances in *E. obliqua* were correlated with seasonal variations, of which temperature variation was particularly important. Blake (1976) also demonstrated an accumulation of inhibitory compounds in the stems of *E. obliqua* seedlings maintained at 30°C for 8 days. Finally, the hastening by elevated temperatures of leaf senescence (Mothes and Baudisch 1958), a process believed to be subject to hormonal regulation, may be interpreted as an effect of temperature on the growth substances involved.

All the evidence discussed above points to the possible existence of qualitative or quantitative differences between species, or between temperature regimes, in the growth substances present in eucalypt seedlings. These substances may be involved in bringing about the effects of high temperature described in Chapters 2 to 4, and differences between species may result in the higher optimum temperature for growth of *E. grandis*. An examination of the growth substances present in

each species at a range of temperatures is therefore likely to yield information on the cause of the differences in performance of eucalypt species in field trials in warm temperate areas.

CHAPTER 6

GROWTH SUBSTANCES IN THE LEAVES AND ROOTS OF *E. GRANDIS* AND *E. REGNANS* SEEDLINGS

6.1. Introduction

In the previous Chapter it was suggested that some qualitative or quantitative difference in the growth substances normally present in *E. grandis* and *E. regnans*, or in their stability to elevated temperatures, may be responsible in part for the different temperature optima for growth of these two species. Alternatively, some of the symptoms of supra-optimal growing temperature described in Chapter 2 may be mediated by growth substance imbalances resulting from different effects of high temperature on membrane permeability, enzyme systems or reaction rates between species.

In either case, an examination of the major growth substances present in each species over a range of temperatures can be expected to provide information which will help to explain the inability of *E. regnans* to survive at temperatures favourable for the growth of *E. grandis*. Such a survey of biologically active substances will reveal the effects of temperature on the range of substances present, and any differences in these effects between the two species. In addition, since *E. regnans* seedlings at 30^{o}/ 25^{o}C grow vigorously for a period before the growth rate begins to decline and symptoms of ill-health appear, a comparison of growth substances in leaves harvested at different times after the

transfer of seedlings to this temperature regime may indicate substances whose concentrations vary with age as well as temperature, such that they are correlated with the seedling growth rate. Substances which show such a correlation may be important in determining the different responses of the two eucalypts to elevated temperatures, and should therefore be subjected to more detailed study.

To examine the growth substance contents of *E. grandis* and *E. regnans* seedlings for this initial purpose, bioassay techniques were used to assess the activity of growth promoters (e.g. gibberellins) and inhibitors in the acid fraction, inhibitors in the neutral fraction, and cytokinins and inhibitors in the aqueous fraction of methanol extracts from leaves and roots, and of promoters and inhibitors in root saps. To determine the effects of temperature on auxin concentrations within each species, indole-3-acetic acid (IAA) was determined in leaf extracts using special extraction and assay techniques; this work is described in Chapter 7.

6.2. Materials and methods

6.2.1. Plant materials

Leaves and roots were collected from the seedlings harvested at intervals for leaf area and fresh weight determination as described in Chapter 2. At each harvest up to 200 g of leaves were collected at random from five seedlings of each species, and 10 g samples drawn from these collections were stored at -20^{o}C until required for extraction. The root systems of the five seedlings were washed free of potting

medium, cut into approximately 3 cm lengths and bulked. 10 g samples of root material were drawn and also stored at -20°C until needed. Root saps were collected from a further five seedlings of each species at each harvest as described in Chapter 4.

6.2.2. Extraction and separation of growth substances

Leaf and root material was extracted with methanol and separated into acid, neutral and aqueous fractions by the process outlined in Figure 6-1. 10 g samples of leaves and roots were homogenised with 70 ml of cold methanol for two minutes at high speed in a Waring blender, and the homogenates stored overnight at 4°C. Solid debris was removed by filtration through Whatman no. 41 paper in a Buchner funnel; the debris was washed with methanol until the leachate was almost colourless. The methanol extract was evaporated to an aqueous residue on a rotary evaporator at 35°C, then taken up in 25 ml of distilled water and adjusted to pH 7 with 10 N KOH.

The aqueous extract was partitioned three times against equal volumes of n-hexane and the organic phases combined to provide a neutral fraction. The aqueous phase was then adjusted to pH 3 with 10 N HCl and partitioned three times against equal volumes of redistilled diethyl ether; the combined organic phases this time provided the acid fraction. Any remaining traces of ether were removed from the aqueous phase by evaporation on a rotary evaporator at 35°C, then this phase was adjusted to pH 7 to provide the aqueous fraction.

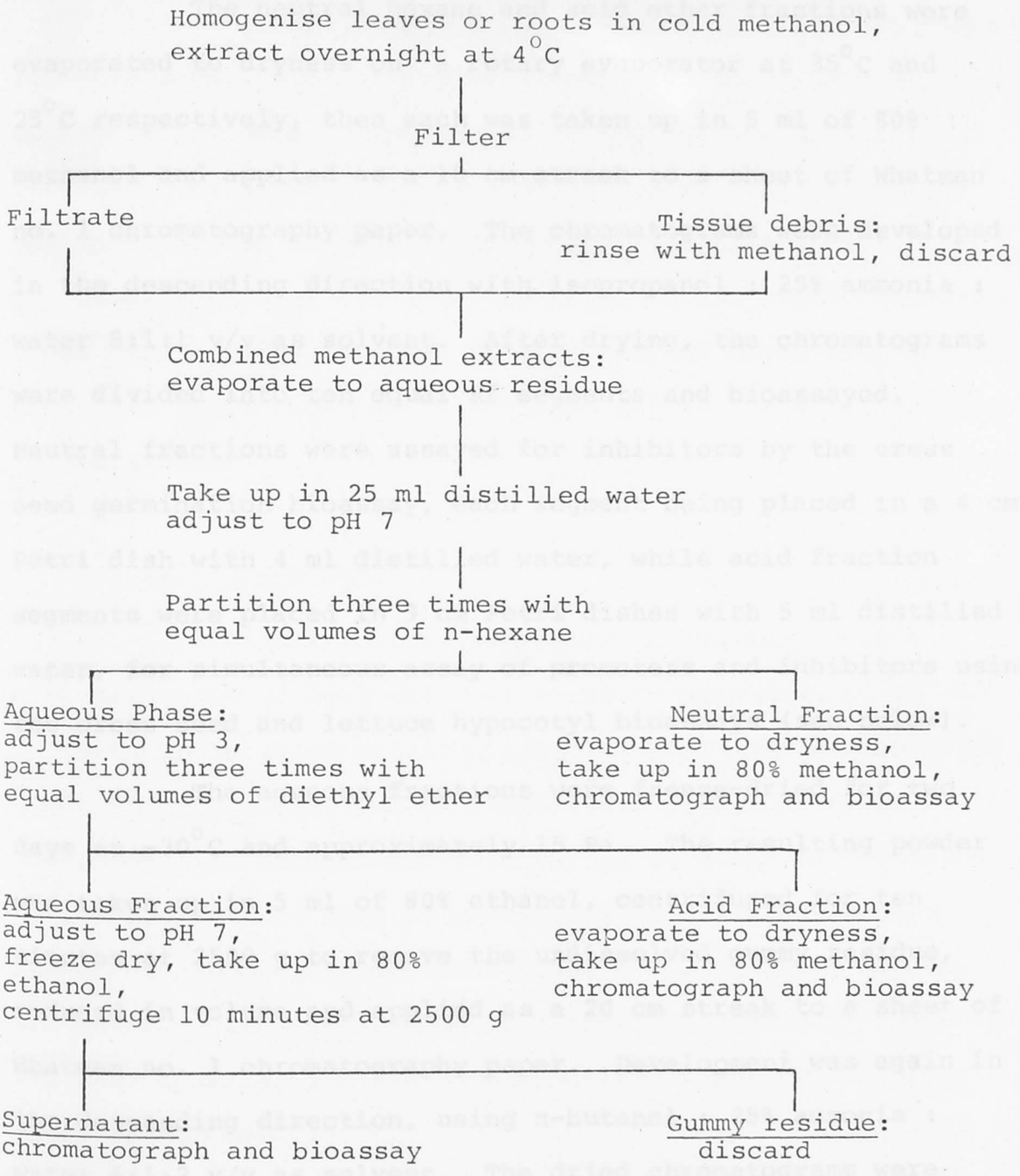


Figure 6-1. Flow diagram showing procedure for extraction and fractionation of eucalypt leaves and roots prior to assay for growth substances.

The neutral hexane and acid ether fractions were evaporated to dryness on a rotary evaporator at 35°C and 25°C respectively, then each was taken up in 5 ml of 80% methanol and applied as a 10 cm streak to a sheet of Whatman no. 1 chromatography paper. The chromatograms were developed in the descending direction with isopropanol : 25% ammonia : water 8:1:1 v/v as solvent. After drying, the chromatograms were divided into ten equal Rf segments and bioassayed. Neutral fractions were assayed for inhibitors by the cress seed germination bioassay, each segment being placed in a 4 cm Petri dish with 4 ml distilled water, while acid fraction segments were placed in 9 cm Petri dishes with 5 ml distilled water, for simultaneous assay of promoters and inhibitors using the cress seed and lettuce hypocotyl bioassays (see below).

The aqueous fractions were freeze-dried for two days at -30°C and approximately 15 Pa. The resulting powder was taken up in 5 ml of 80% ethanol, centrifuged for ten minutes at 2500 g to remove the undissolved gummy residue, reduced in volume and applied as a 20 cm streak to a sheet of Whatman no. 1 chromatography paper. Development was again in the descending direction, using n-butanol : 25% ammonia : water 6:1:2 v/v as solvent. The dried chromatograms were again divided into ten equal Rf segments which were either placed in 4 cm Petri dishes with 4 ml of distilled water for cress seed bioassay or in 9 cm dishes with 5 ml of phosphate-tyrosine buffer for *Amaranthus* betacyanin bioassay (see below).

Root saps were not partitioned, but chromatographed as whole saps. 6 ml of sap was evaporated to dryness at 35°C on a rotary evaporator, taken up in 4 ml of 80% methanol and streaked over 20 cm on Whatman no. 1 paper. Chromatography was again in the descending direction with isopropanol : 25% ammonia : water 8:1:1 v/v as solvent; the dried chromatograms were divided into ten Rf segments and bioassayed for promoters and inhibitors in the same way as the acid fraction chromatograms.

6.2.3. Bioassays

Gibberellin-like promoters in the root saps and acid fractions of leaf and root extracts were assayed by the lettuce hypocotyl elongation test (Frankland and Wareing 1960). Seeds of lettuce (cv. Great Lakes) were placed on moist filter paper and left in darkness at 25°C for 24 hours to germinate. At the end of this time 10 germinates of uniform size were placed in each Petri dish containing a chromatogram segment and water. With each set of 50 dishes containing Rf segments, 4 dishes containing blank chromatography paper and water were included as controls.

The dishes were placed under fluorescent light in a controlled environment cabinet maintained at 25°C for four days. Occasionally it was necessary to add a little extra water to dishes which showed signs of drying out before this time. The length of each lettuce hypocotyl was then measured to ±0.5 mm and the results averaged for each dish, expressed as a percentage of the control mean and histogrammed. The 95%

confidence limits for the control mean were calculated, and individual dish means which fell within these limits were not regarded as significant responses. A dose-response curve for this bioassay was prepared by assaying gibberellic acid (GA_3) solutions of a range of concentrations, and is shown in Figure 6-2.

Inhibitors in the root saps and all fractions of the leaf and root extracts were assayed by the cress seed germination bioassay, as used by Paton *et al.* (1970). For this, ten cress seeds were placed in each Petri dish containing a chromatogram segment and water, using blank chromatography paper for controls and including 8 control dishes with each batch of 50 Rf segments as before. The dishes were kept at 25°C in the light for 4 days, after which the number of germinated seeds in each dish was recorded. The control mean and 95% confidence limits were calculated from the results of all control dishes included with each batch of assays. As the confidence intervals obtained were invariably less than ±1 germinate per dish, an interval of ±1 was assumed and dishes in which the number of germinates differed from the mean by 2 or more were considered as showing significant responses. A dose-response curve for abscisic acid (ABA) in the cress seed bioassay is shown in Figure 6-3.

The leaf and root aqueous fractions were assayed for cytokinins by a method similar to Biddington and Thomas' (1973) modification of Bigot's (1968) *Amaranthus* betacyanin bioassay. Seeds of *Amaranthus tricolor* cv. Perfecta were placed on moist filter paper and allowed to germinate in darkness at 25°C for three days. The seed coats were removed with

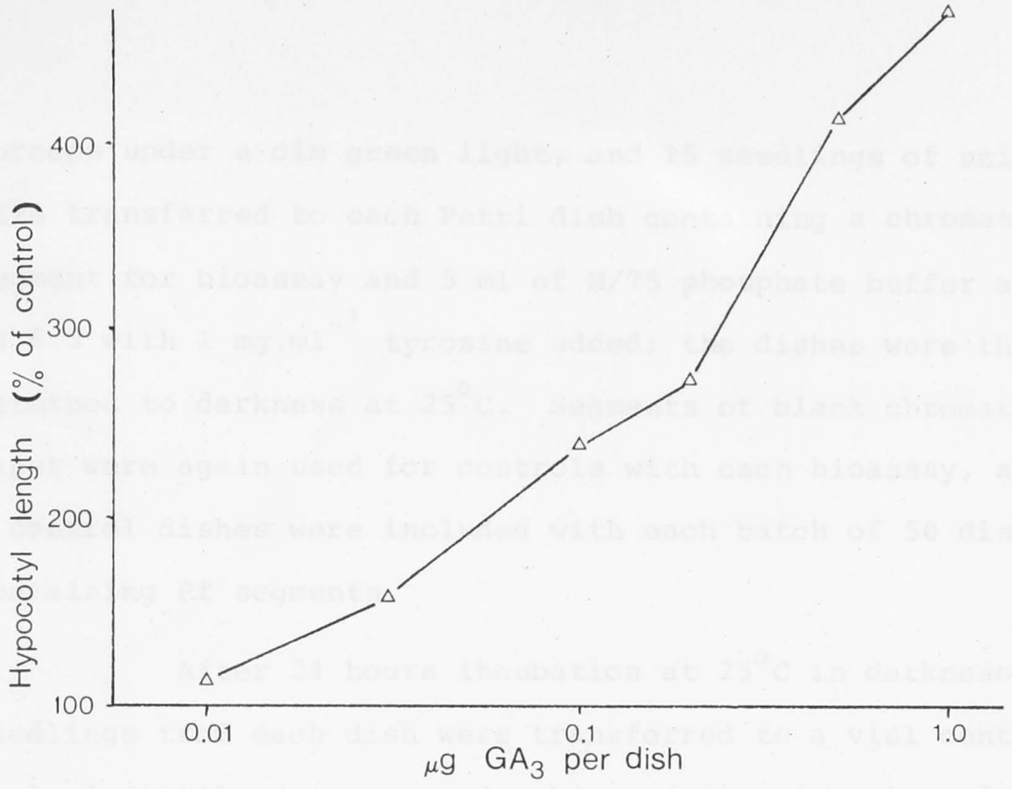


Figure 6-2. Dose-response curve for gibberellic acid (GA_3) in the lettuce hypocotyl elongation bioassay. Each point is the mean of four assays, using 10 lettuce germinates per dish.

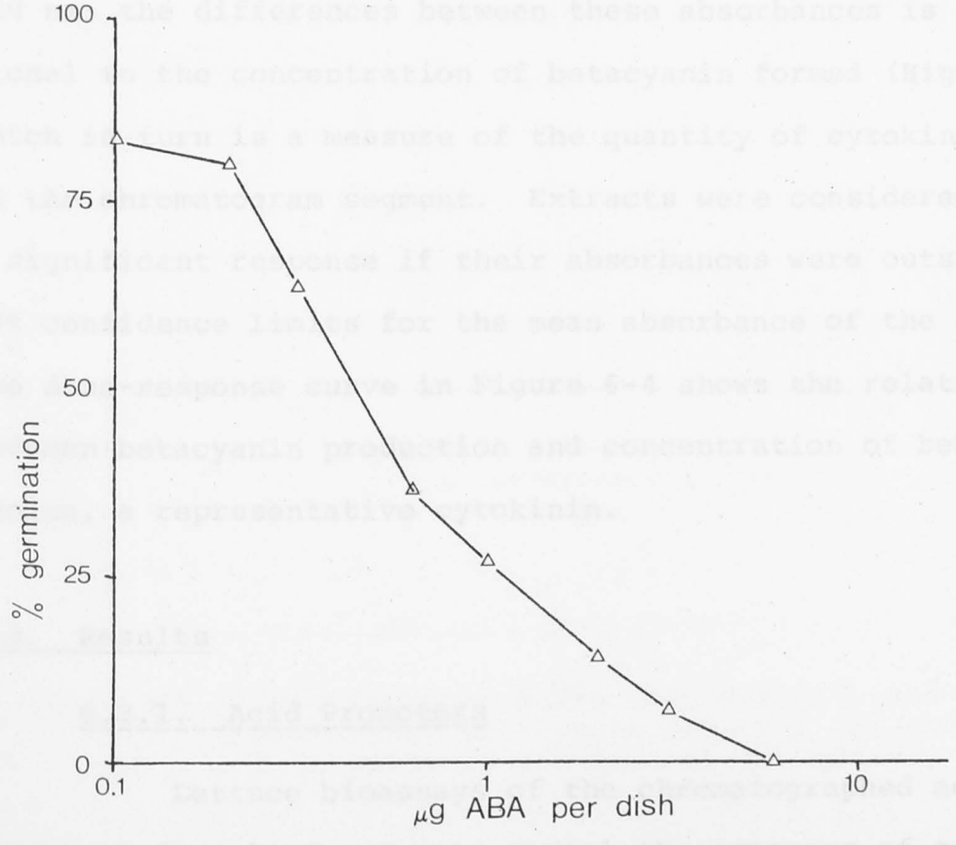


Figure 6-3. Dose-response curve for Abscisic Acid in the cress seed germination bioassay. Each point is the mean of two assays, using 15 cress seeds per dish.

forceps under a dim green light, and 15 seedlings of uniform size transferred to each Petri dish containing a chromatogram segment for bioassay and 5 ml of M/75 phosphate buffer at pH 6.3 with 1 $mg.ml^{-1}$ tyrosine added; the dishes were then returned to darkness at 25°C. Segments of blank chromatography paper were again used for controls with each bioassay, and 4 control dishes were included with each batch of 50 dishes containing Rf segments.

After 24 hours incubation at 25°C in darkness, the seedlings from each dish were transferred to a vial containing 3 ml of distilled water, and subjected to two cycles of freezing and thawing to extract betacyanin from the cotyledons. The absorbance of each extract was measured at 542 nm and 620 nm: the differences between these absorbances is proportional to the concentration of betacyanin formed (Bigot 1968), which in turn is a measure of the quantity of cytokinin present in the chromatogram segment. Extracts were considered to show a significant response if their absorbances were outside the 95% confidence limits for the mean absorbance of the controls. The dose-response curve in Figure 6-4 shows the relation between betacyanin production and concentration of benzyladenine, a representative cytokinin.

6.3. Results

6.3.1. Acid Promoters

Lettuce bioassays of the chromatographed acid fractions from leaf extracts reveal the presence of considerable growth promoting activity, mostly confined to three zones of the chromatograms (Table 6-1). These zones are broad and

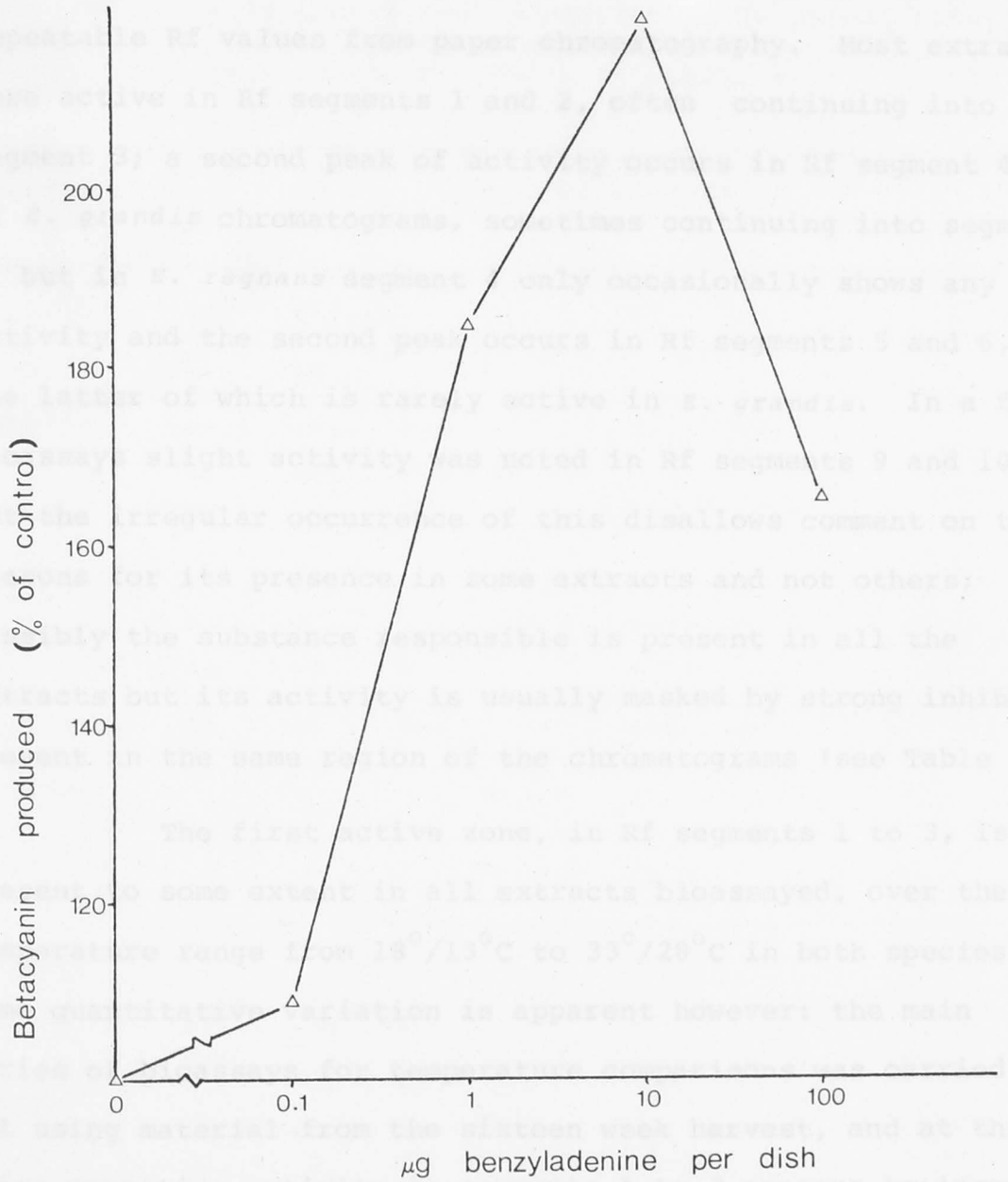


Figure 6-4. Dose-response curve for benzyladenine in the *Amaranthus* betacyanin bioassay for cytokinins. Each point is the mean of two assays.

may overlap, due to the likely presence of more than one active compound in each zone and the difficulty of obtaining repeatable Rf values from paper chromatography. Most extracts were active in Rf segments 1 and 2, often continuing into segment 3; a second peak of activity occurs in Rf segment 4 of *E. grandis* chromatograms, sometimes continuing into segment 5, but in *E. regnans* segment 4 only occasionally shows any activity and the second peak occurs in Rf segments 5 and 6, the latter of which is rarely active in *E. grandis*. In a few bioassays slight activity was noted in Rf segments 9 and 10, but the irregular occurrence of this disallows comment on the reasons for its presence in some extracts and not others; possibly the substance responsible is present in all the extracts but its activity is usually masked by strong inhibitors present in the same region of the chromatograms (see Table 6-3).

The first active zone, in Rf segments 1 to 3, is present to some extent in all extracts bioassayed, over the temperature range from $18^{o}/13^{o}C$ to $33^{o}/28^{o}C$ in both species. Some quantitative variation is apparent however: the main series of bioassays for temperature comparisons was carried out using material from the sixteen week harvest, and at this stage promoting activity in segments 1 to 3 appears to increase with rising temperature up to a maximum at $24^{o}/19^{o}C$ in both species. Activity at $30^{o}/25^{o}C$ is considerably reduced; this was confirmed by performing the extraction and bioassay of the $24^{o}/19^{o}C$ and $30^{o}/25^{o}C$ material in triplicate. At $33^{o}/28^{o}C$ no further reduction in activity is apparent.

Table 6-1. Results of the lettuce hypocotyl elongation bioassay for growth-promoting substances in leaf acid fractions.
+ : significant promotion at 5% probability (110-130% of control);
++ : 130-160%; +++ : 160-200%; ++++ : >200%.

Seedling Source	Rf Segment 1	2	3	4	5	6	7	8	9	10
R16/18*	++	+++			+	++				
R16/21	+	++								
R16/24	++++	++++	+++		+	+	++			
	++	+++	+++	++						
	+++	++	+							
R16/30	+++	++	+		++	++				++
	+	+			+	++++				+
	++	++	+			+				
R16/33	++	++	++			+++	+			
R20/24		+		+	+					
R20/30	++++	+++								
	++	++								
G16/18	++									+
G16/21	++++	+++	+							
G16/24	+++	+++	++	+++						
	++++	++	++	+						
	++++	+++	++	++	+++	+				
G16/30	+	+		+						
			++		+					
	++	+	+							
G16/33	+++	++				+			+	
G10/30	+++	+	+	++	++					
G10/33	+++	+		+	+					

* The notation "R16/18", "G10/30" etc. in this and subsequent Tables to describe the source of extracts indicates, e.g. *E. regnans*, 16 week harvest, day temperature 18°C; similarly *E. grandis*, 10 week harvest, day temperature 30°C; and so on.

To check for seedling age effects on promoter concentrations, some material from the ten week harvest of *E. grandis* and the twenty week harvest of *E. regnans* was also assayed. Little or no change in promoting activity of the first active zone from *E. grandis* leaves occurs between ten and sixteen weeks at $33^{\circ}/28^{\circ}C$ but there is some evidence of a decline in activity with age at $30^{\circ}/25^{\circ}C$. In *E. regnans* the activity of $24^{\circ}/19^{\circ}C$ material in this zone appears to decrease between sixteen and twenty weeks, while the activity of $30^{\circ}/25^{\circ}C$ material remains constant.

The second active zone, in segments 4 and 5, appears to be caused by promoters absent from *E. regnans*: activity in segment 4 from *E. regnans* extracts was found in only two bioassays, where it was most likely associated with activity in segment 3 or 5. Promotion in this zone in *E. grandis* leaves at the sixteen week harvest was considerable at $24^{\circ}/19^{\circ}C$, weak at $30^{\circ}/25^{\circ}C$ and absent at higher and lower temperatures. At the ten week stage it was moderately strong at $30^{\circ}/25^{\circ}C$ and still present at $33^{\circ}/28^{\circ}C$. These results suggest the presence of a growth promoting compound at $24^{\circ}/19^{\circ}C$ and above, the activity of which decreases as the seedlings grow older; the decline in activity appears to begin sooner or proceed more rapidly at higher temperatures.

In bioassays of *E. regnans* leaf extracts a third active zone in Rf segments 5 to 7 (and centering on Rf 6) is important, but only two of the *E. grandis* bioassays showed any activity in segments 6 or 7, and at least one of these is clearly due to the strong zone two promoter in segment 5. In

E. regnans leaves from the sixteen week harvest, some activity was found in this third zone at 18°/13°C and 24°/19°C, but strong promotion occurred at 30°/25°C and 33°/28°C. At the 20 week stage promotion was still present in the 24°/19°C extract but could not be detected in 30°/25°C material. This complete disappearance of a strong promoter with increasing seedling age at 30°/25°C (but not lower temperatures) coincides with the onset of stress symptoms in *E. regnans* at this temperature.

Table 6-2 shows the results of lettuce bioassays of chromatographed acid fractions from roots. The concentrations of growth promoting substances here were clearly much lower than in the leaves, and in some extracts were too low for detection by the lettuce bioassay. As a result, comparisons between bioassays can only be tentative, and the leaf data provide a far better basis for study of the effects of temperature and age on promoter concentrations in each species.

Activity in zone 1 (Rf 1 to 3) and zone 2 (Rf 4 and 5) is present sporadically in both species, but no activity was detected in zone 3 (Rf 6 and 7) from *E. regnans* root extracts at any temperature, and it appears that the promoter responsible for activity in this zone of leaf extracts is absent from the roots. Unlike the leaf extracts of this species, some promotion independent of zones 1 and 3 was found in zone 2 (Rf segments 4 and 5): it may be that part of the activity attributed to zones 1 and 3 in the leaf extracts was in fact due to relatively low concentrations of the zone 2 promoters.

Table 6-2. Results of the lettuce hypocotyl elongation bioassay for growth-promoting substances in root acid fractions. For explanation of symbols see Table 6-1.

Seedling Source	Rf Segment 1	2	3	4	5	6	7	8	9	10
R16/18	++	+	++	+	+					
R16/21	+		+							
R16/24										
R16/30	++	++	+	+						
R16/33									+	
R20/24	++			++	+					
R20/30	++		+							
G16/18					+					
G16/21										
G16/24	+	+			+					
G16/30										
G16/33		+		+	+					
G10/30	++		+	++						
G10/33	+	+								

6.3.2. Acid inhibitors

Bioassays of leaf acid fractions by the cress germination test revealed strong inhibition in all extracts of both species, mostly confined to Rf segments 7 to 9 (Table 6-3). In segments 1 to 4 only slight inhibition occur-

red, mostly in *E. grandis* extracts at the lower temperatures: this may be a direct effect of sub-optimal temperature in this species, absent from *E. regnans* due to its lower temperature optimum, but it is probably not associated with the difference in high temperature responses investigated in this study. Slight inhibition in segments 2 or 3 was also seen in both species at $33^{\circ}/28^{\circ}C$ (in *E. grandis* at ten weeks but not sixteen).

The most interesting species difference appears in Rf segments 5 and 6. In *E. grandis* inhibition occurs in this zone only at $18^{\circ}/13^{\circ}C$ and $21^{\circ}/16^{\circ}C$, which may again be related to the effects of "low" temperature on the growth of this species. Although slight inhibition occurred in segment 5 of the *E. regnans* $21^{\circ}/16^{\circ}C$ extract, it appears to be weaker than in *E. grandis*, and the major segment 5-6 inhibition in this species occurs at $30^{\circ}/25^{\circ}C$. This is weak at sixteen weeks but much stronger by twenty weeks, again correlating with the reduction in growth rate and onset of stress symptoms in *E. regnans* at this temperature. The inhibitor is however absent from the $33^{\circ}/28^{\circ}C$ extract.

As the occurrence of this Rf 0.4-0.6 inhibitor in *E. regnans* but not *E. grandis* may be related to the difference in behaviour of the two species at supra-optimal temperatures, an attempt was made to separate it from a 30 g sample of leaves from the twenty-week harvest of *E. regnans* seedlings grown at $30^{\circ}/25^{\circ}C$. The acid fraction was extracted and chromatographed on Whatman 3MM paper in isopropanol : ammonia : water 8:1:1

v/v, and a section of the chromatogram equivalent to 10 g fresh weight of leaves bioassayed as usual to check for the presence of the inhibitor. Inhibition was found in segment 4 (higher than previously, due to the heavier paper used) and in segments 7 to 10.

The Rf 0.3-0.4 zone from the remainder of the chromatogram (equivalent to 20 g fresh weight of tissue) was eluted with 40 ml of wet ethyl acetate, centrifuged briefly to remove paper fibres, and evaporated to dryness. Column chromatography on insoluble polyvinylpyrrolidone (PVP) was used to separate the inhibitor from other compounds present on the chromatogram. A 20 X 1.6 cm column of PVP was prepared as described by Glenn *et al.* (1972) and the eluate, taken up in 3 ml of 0.1 M phosphate buffer at pH 8.0, loaded onto it. The column was eluted with the same buffer and fifteen 10 ml fractions were collected. These were adjusted to pH 3.0 with 10 N HCl and extracted three times with equal volumes of ethyl acetate. The combined organic phases were allowed to evaporate to dryness on filter paper in Petri dishes, then tested for inhibiting activity by the cress bioassay. Four distinct inhibiting compounds were located in this way, indicating that the inhibition present at Rf 0.4-0.6 in chromatographed *E. regnans* leaf fractions from $30^{\circ}/25^{\circ}$C is in fact due to a complex of inhibitory substances. More work is therefore needed to establish which if any of these are involved in the effects of high temperature on the growth of this species.

Table 6-3. Results of the cress seed germination bioassay for growth-inhibiting substances in leaf acid fractions.
+ : significant inhibition at 5% probability (50-60% germination);
++ : 30-40%; +++ : 10-20%; ++++ : 0%.

Seedling Source	Rf Segment 1	2	3	4	5	6	7	8	9	10
R16/18							++++	+++	++++	
R16/21					+		+++	++++	++++	+
R16/24							+	++++	++++	+++
								+++	++++	
							++	+++	++++	++
R16/30						+	++++	++++	++++	
						+	++++		++++	
					+		+	++++	++++	++
R16/33			+				+++	++++	++++	++++
R20/24							++	++	+	
R20/30				+	++	++++	++++		+++	
					+	+	++++	++++	++++	+++
G16/18		+				++	++++	++++	++	+
G16/21		+				++	++++	+++	++	
G16/24			+	+			++++	++++	++++	++
		+					++	++++	+++	
				+			++++	++++	++++	
G16/30							++	++++	+++	++
							++++	++++	++++	
							++++	++++	+++	++
G16/33							++++	++++	++++	
G10/30							++	+	++++	
G10/33		+					++++	+	++++	

The severe inhibition in Rf segments 7 to 10 from both species is obviously also caused by more than a single compound, and in some cases two zones can be discerned, with inhibition in segment 8 absent or weak relative to the adjacent segments. Little difference between species, temperatures or seedling ages is apparent in these zones, nor can any correlation with the growth behaviour of the seedlings be found. However, if a number of inhibitory substances of similar chromatographic properties are present it is conceivable that even large variations in the concentrations of some of them could be masked by the presence of the others.

To separate the inhibiting compounds of this complex, the acid fractions from 10 g samples of leaves from the 20 week harvest of *E. regnans* at $18^{o}/13^{o}$C and $21^{o}/16^{o}$C were extracted and chromatographed as before. Half of each chromatogram was bioassayed with cress seed to confirm that inhibition was present in the extract at Rf 0.6-1.0. Two adjoining inhibitory zones were again found in this region; inspection of the remaining chromatograms in ultraviolet light revealed red and yellow fluorescent bands associated with the inhibition at Rf 0.65-0.8 (inhibitor A) and a yellow-orange band associated with the inhibition at Rf 0.85-1.0 (inhibitor B). These two zones were eluted separately from each chromatogram with 25 ml of 80% methanol.

The eluates were reduced in volume on a rotary evaporator and streaked onto silica gel HF_{254} plates for thin layer chromatography (TLC), using chloroform : ethyl acetate : acetic acid 60:40:5 v/v as solvent. After drying, the plates

were examined under UV light and quenching bands marked. One plate of each inhibitor from each temperature was then divided into ten Rf segments, each of which was eluted with 5 ml of 80% methanol. The eluates were allowed to evaporate to dryness on filter paper in 4 cm Petri dishes, then 4 ml of distilled water and 15 cress seeds were placed in each dish for bioassay of the inhibitors.

The results from both temperature regimes were the same: the inhibitor A plates had inhibitory zones at Rf 0.4-0.5 (A1) and Rf 0.7-0.8 (A2) while the B plates were inhibitory at Rf 0-0.1 (B1) and Rf 0.9-1.0 (B2); only the last of these inhibiting zones corresponded to a UV quenching band. These four zones were scraped from the remaining plates and eluted with 80% methanol, then reduced in volume and streaked again on silica gel HF_{254} plates for TLC with hexane:ethyl acetate 1:1 v/v as solvent. After drying, the plates were divided into Rf segments, eluted and bioassayed as before.

Three inhibitory zones were located on the A1 plates, in Rf segments 1, 5 and 8; another three were found on the B2 plate, in segments 1, 3 and 10; and the A2 and B1 plates each contained a single inhibitor, in segments 7 and 2 respectively. The two stage TLC procedure was thus very successful in separating the inhibitor complex found on paper chromatograms into as many as eight distinct compounds with growth-inhibiting properties; further purification of these might reveal even more. Because of the large number of compounds in the complex, and the lack of any apparent overall correlation with the effects of temperature on growth, this work was not continued further and the identity of the compounds remains unknown. A

large number of acidic inhibitors are known, many of which (e.g. phenolic acids of the benzoic and cinnamic acid families) could be expected to run between Rf 0.6 and 1.0 in isopropanol : ammonia : water 8:1:1.[1]

Table 6-4. Results of the cress seed germination bioassay for growth-inhibiting substances in root acid fractions. For explanation of symbols see Table 6-3.

Seedling Source	Rf Segment 1	2	3	4	5	6	7	8	9	10
R16/18				+		+	+		+	
R16/21				+			+	+	+	
R16/24	+				+		+		+	
R16/30		+	+						++	
R16/33							+	+		
R20/24	+									
R20/30									+	
G16/18	+	+					++		++	
G16/21										
G16/24	+		+			+				+
G16/30	+	+			+					+
G16/33						++			+	
G10/30						+				
G10/33			+						++	

[1] Dr. Brian Ferguson, of the Australian National University, Department of Chemistry, has attempted a similar separation and characterisation of the acid inhibitor complex from leaves of *E. grandis*. Mass spectra of several compounds active in the cress seed germination test have been obtained, but do not match the spectra of common acidic inhibitors and the compounds have not yet been identified. The results obtained do indicate that activity in the acid fractions of these leaves is due to distinctly acidic compounds and not simply to the incomplete removal of neutral inhibitors known to be present in *E.grandis* leaves.

Cress bioassays of acid fractions extracted from the roots (Table 6-4) reveal only weak inhibition, occurring sporadically in all segments of the chromatograms. As the active substances present are obviously in low concentration or are only weakly inhibitory, it is probable that in some extracts they were present but went undetected by the bioassay procedure used, resulting in the seemingly anomalous gaps of Table 6-4 which make detailed comparisons between bioassays hazardous.

As in the leaf acid fractions, inhibition is again present in both species, though relatively weak, in segments 9 and 10, but the segment 7 to 8 inhibitors were not found in *E. grandis* extracts except at $18^{o}/13^{o}C$ - this may be due to the same substance which caused inhibition in segment 6 of *E. grandis* leaf acid fractions at low temperatures. The Rf 0.4-0.6 inhibitor important in *E. regnans* leaf extracts is virtually absent from the roots, slight inhibition being found only at $18^{o}/13^{o}C$ and $24^{o}/19^{o}C$; consistent inhibition is present in this zone on *E. grandis* chromatograms where it is confined to the upper temperatures. The weak inhibition in segments 1 to 4 of both species is unlikely to be of importance to this study; in *E. grandis* it follows much the same pattern as in the leaf extracts, except that it is present right up to $30^{o}/25^{o}C$.

6.3.3. Neutral inhibitors

Table 6-5 shows the results of cress seed bioassays of chromatographed neutral fractions of leaf extracts. The overall pattern of inhibition is similar to that seen in the acid inhibitor bioassays: strong inhibition extending over

several Rf segments at the bottom of the chromatogram, with occasional weak activity nearer the top. It must be realised that the partitioning schedule used cannot be ideal for isolation of all the chemically diverse compounds present in plant extracts, so it is quite possible for the same substance to be responsible for inhibiting activity in both the acid and neutral fractions. However, the complex of "neutral" inhibitors in segments 7 to 10 appears to differ from the "acid" complex: inhibition is much stronger and more consistently present in segment 10, no tendency to separate into two zones is apparent, and although segment 7 is again strongly active in *E. regnans* leaf extracts, it is never so in those from *E. grandis*.

The generally weak and inconsistent inhibition located in segments 1 to 6 from both species shows little relation to temperature or species effects: as in the acid fractions there is some evidence of increasing inhibition in *E. grandis* at low temperatures, and both species again show some inhibition at $33^{\circ}/28^{\circ}$C in the vicinity of Rf segment 3. The main block of inhibitors on *E. regnans* chromatograms shows no variation with temperature or age that cannot be ascribed to small differences in Rf of the active substances between chromatograms, but in *E. grandis* the segment 7-8 inhibition present at high temperatures at ten weeks is gone or much reduced by sixteen weeks. The segment 9-10 inhibition is unaffected by temperature or age, as in *E. regnans*. Paton *et al.* (1970) and Crow *et al.* (1977) reported the presence of a group of novel inhibitors from this zone of chromatographed neutral fractions extracted from mature leaves of *E. grandis*; the

results obtained here suggest that *E. regnans* leaves may also contain these inhibitors or related compounds. The findings of these authors also emphasise the suggestion that inhibition in a given zone of the chromatograms bioassayed is often due to a complex of substances which may be closely related chemically, rather than to a single inhibitor.

Table 6-5. Results of the cress seed germination bioassay for growth-inhibiting substances in leaf neutral fractions. For explanation of symbols see Table 6-3.

Seedling Source	Rf Segment 1	2	3	4	5	6	7	8	9	10
R16/18							++++	++++	++++	++++
R16/21		+			+			++++	++++	++++
R16/24					+		++++	++++	++++	++++
R16/30							++++	++++	++++	+++
R16/33			+				+++	++++	++++	++++
R20/24							+++	++++	++++	++++
R20/30	+						+	++++	++++	++++
G16/18	++	+					+		++++	+++
G16/21		+				+		+	++++	+
G16/24								+	++++	+++
G16/30									++++	++
G16/33								+	++++	++++
G10/30		+						++++	++++	++++
G10/33				+			+	++++	++++	++++

Table 6-6. Results of the cress seed germination bioassay for growth-inhibiting substances in root neutral fractions. For explanation of symbols see Table 6-3.

Seedling Source	Rf Segment 1	2	3	4	5	6	7	8	9	10
R16/18		+							+	
R16/21					+			+	+++	
R16/24	+								++	+
R16/30					+				+	
R16/33		+							+	
R20/24								+	+	
R20/30								++		
G16/18			++						+	
G16/21					+		+		++	
G16/24		++	+		+	++		+	++	+
G16/30					+	+			+	+
G16/33			+					++	++	++
G10/30										
G10/33									++	

Table 6-6 shows the results of cress bioassays of chromatographed neutral fractions from root extracts. As with the acid fractions, inhibition in the root material is generally weaker than in the leaves. Only Rf segment 9 retains consistent inhibiting activity, occasionally extending into segments 8 and 10. The segment 7 inhibitor of the leaf neutral fractions is absent, but more activity is present in segments

1 to 6, at least in *E. grandis* extracts. This appears to occur in two zones, in segments 1 to 3 and 5 to 6. Little can be inferred from the weak and sporadic occurrences of inhibition in these zones, but they are present in extracts of both species (possibly at higher concentrations in *E. grandis*) and while the segment 1-3 inhibition occurs over the whole range of temperatures studied the segment 5-6 inhibition was not found at either the highest or the lowest temperature.

6.3.4. Aqueous inhibitors

Blake (1976) studied the effects of relatively short periods of exposure to moderately high temperatures ($30^{\circ}C$) on seedlings of *E. obliqua*; this species was not among those tested at Coffs Harbour (Pryor 1972), but is closely related to *E. regnans* and *E. fastigata* Deane and Maiden, both of which failed there. Among other effects, Blake noted the presence of an inhibitor which increased in concentration over the eight-day period of study, with a simultaneous increase in the concentration of phenolic compounds. These results support an earlier suggestion (Blake 1973) that high concentrations of phenolic glycosides in *E. obliqua* seedlings during summer may partly explain the seasonal inhibition of growth observed in this species. As such a high-temperature inhibition resembles that observed in this study, the possibility of high concentrations of phenolic glycosides building up in older seedlings of *E. regnans* was investigated by assaying for inhibitory activity in the aqueous fractions.

As a first step, the aqueous fractions of leaf and root extracts from *E. regnans* at the twenty-week stage ($24^{\circ}/19^{\circ}C$ and $30^{\circ}/25^{\circ}C$) and *E. grandis* at the ten-week stage ($30^{\circ}/25^{\circ}C$ and $33^{\circ}/28^{\circ}C$) were tested for inhibiting activity by the cress bioassay, without chromatographic separation. The freeze-dried aqueous fractions were taken up in 5 ml of 80% ethanol and the solutions allowed to evaporate to dryness on filter paper in Petri dishes. The ethanol-insoluble gummy residues were taken up in 4 ml of distilled water and also transferred to Petri dishes containing filter paper, then fifteen cress seeds were placed in each dish for bioassay as usual.

All extracts showed strong inhibition, both in the ethanol-soluble and -insoluble parts; only in the ethanol-soluble root aqueous fractions was the inhibition of cress seed germination less than 100%, indicating that in this as well as the acid and neutral fractions the inhibitors tend to be concentrated in the leaves rather than the roots. On the basis of these results, the ethanol-soluble parts of freeze-dried leaf aqueous fractions from the same harvests were chromatographed and bioassayed as described in Section 6.2; the results of these bioassays are histogrammed in Figure 6-5.

It can be seen that the major inhibition of the leaf aqueous fractions occurs in Rf segments 6 and 7 in both species, and may be present in higher concentrations in *E. grandis* at 10 weeks than in *E. regnans* at 20 weeks, with little or no difference between the temperatures tested. There is no evidence therefore that the decline in growth rate and other stress symptoms observed in *E. regnans* after 16 weeks at $30^{\circ}/25^{\circ}C$ is caused by an increase in an aqueous inhibitor at Rfs 6 and 7

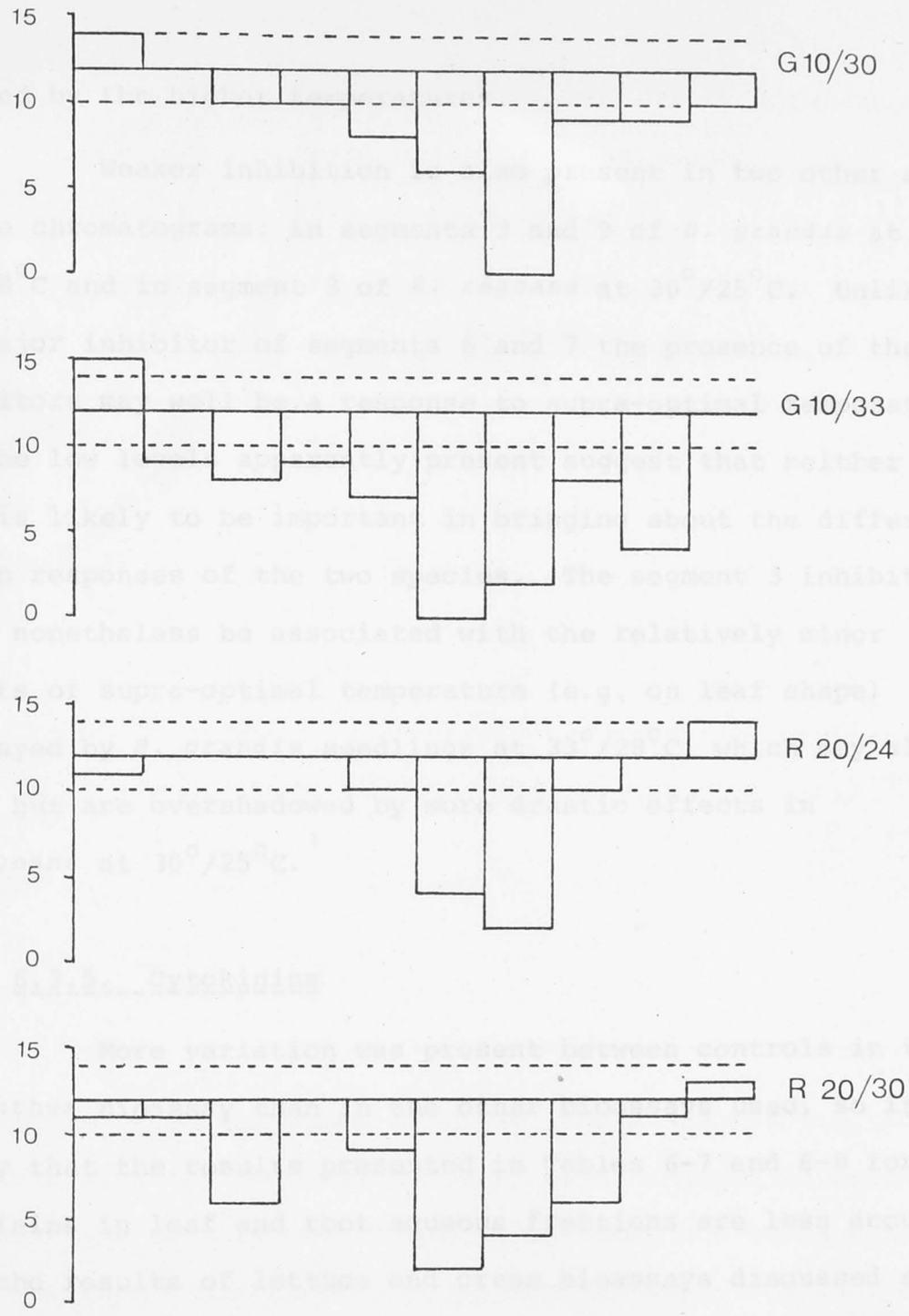


Figure 6-5. Histogrammed results of cress germination bioassays of chromatographed leaf aqueous fractions.
Abscissa - Rf segments 1 to 10; Ordinate - number of germinates. Dashed lines show 95% confidence limits for the control mean.

induced by the higher temperatures.

Weaker inhibition is also present in two other areas of the chromatograms: in segments 3 and 9 of *E. grandis* at $33^{o}/28^{o}$C and in segment 3 of *E. regnans* at $30^{o}/25^{o}$C. Unlike the major inhibitor of segments 6 and 7 the presence of these inhibitors may well be a response to supra-optimal temperature, but the low levels apparently present suggest that neither of them is likely to be important in bringing about the differing growth responses of the two species. The segment 3 inhibitor could nonetheless be associated with the relatively minor effects of supra-optimal temperature (e.g. on leaf shape) displayed by *E. grandis* seedlings at $33^{o}/28^{o}$C, which may also occur but are overshadowed by more drastic effects in *E. regnans* at $30^{o}/25^{o}$C.[1]

6.3.5. Cytokinins

More variation was present between controls in the *Amaranthus* bioassay than in the other bioassays used, so it is likely that the results presented in Tables 6-7 and 6-8 for cytokinins in leaf and root aqueous fractions are less accurate than the results of lettuce and cress bioassays discussed so far. Considerable differences between species can nevertheless

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[1] E.P. Bachelard in subsequent work (unpublished) has obtained preliminary evidence that high temperatures can result in the appearance of a larger amount of aqueous inhibitors (Rf^{S} 2, and 6-8 in isopropanol:ammonia:water 8:1:1) in *E. regnans* than in *E. grandis* after 16 weeks. This may yet prove to be of importance in the observed growth responses of the two species.

Table 6-7. Results of the *Amaranthus* betacyanin bioassay for cytokinins in leaf aqueous fractions.
+ : significant activity at 5% probability (117-130% of control);
++ : 130-160%; +++ : > 160%.

Seedling Source	Rf Segment 1	2	3	4	5	6	7	8	9	10
R16/18	+			++	+	++	+			+
R16/21										
R16/24	++		++	++	++		++	+	+	
R16/30	++		++	++			++	++	+	+++
R16/33	++	+	++	+++	+					
G16/18										++
G16/21										++
G16/24										
G16/30										++
G16/33										

be seen: in chromatographed *E. grandis* leaf aqueous fractions, cytokinin activity was detected only in Rf segment 10, but in *E. regnans* activity was found in at least one extract in every segment. This appears to be concentrated into four zones, namely segments 1-2, 3-5, 6-8 and 9-10. Making allowance for the probable inaccuracy of the bioassay results, there is little evidence of difference in cytokinin concentrations between temperature regimes, except for the absence of activity from segments 6-10 at $33^{\circ}/28^{\circ}$C.

Table 6-8. Results of the *Amaranthus* betacyanin bioassay for cytokinins in root aqueous fractions. For explanation of symbols see Table 6-7.

Seedling Source	Rf Segment 1	2	3	4	5	6	7	8	9	10
R16/18			++					++		+
R16/21		++	++					+		++
R16/24		+++							++	++
R16/30	++				++	+	++		+	
R16/33		+	++	++	++		+++			
G16/18	+++			+			+			
G16/21	+			+					+	
G16/24	+++					++				
G16/30		++	++	+		++				
G16/33	+	+++	++		+		+	+++		

Activity is more evenly distributed between species in the root extracts, but again all Rf segments show some activity. It is difficult to define zones of activity from the bioassay responses obtained, and difficult as a result to make comparisons between species or temperatures. The largest species difference is the activity at low temperatures in *E. regnans* but not *E. grandis* in Rf segments 8 to 10, and the presence of activity at high temperatures in both species in segments 5 and 6 may represent a temperature effect. In no case was a distinct species - temperature interaction observed and hence there is no basis for considering the differences in species response to temperature to be caused by cytokinin levels.

6.3.6. Promoters and inhibitors in root saps

Comparison of Tables 6-9 and 6-10 showing activity of root saps with the results presented earlier on promoting and inhibiting activity of acid and neutral fractions from root extracts indicates that while most of the inhibiting substances present in the roots are held within the cells, the promoters move freely in the sap. The inhibitor detected in Rf segment 4 of *E. grandis* 33°/28°C sap coincides with a fluorescent band not seen on chromatograms of the other saps, and also with inhibition in the acid fraction of a root extract from the same harvest (Table 6-4).

Table 6-9. Results of the lettuce hypocotyl elongation bioassay for growth-promoting substances in root saps. For explanation of symbols see Table 6-3.

Seedling Source	Rf Segment 1	2	3	4	5	6	7	8	9	10
R30/18	+	++	+	+	++		++	+		
R30/21	+++	+		+	+	+		+		
R30/24	+++				+	+				
R30/30	++	+++								
G16/18	++			++	+				+	
G16/21	+	+	++		+	+++	+	++		
G16/24	+	+	+	++	++	+	+		+	+
G16/30	+++	+		+	++	+			+	
G16/33	+++	++	+	+	+	+++	++			++

The root sap promoters are present in at least some saps in all ten Rf segments, representing a wider range of

compounds than were detected even in the leaf acid fractions. This may result from the masking of promoting activity in the leaf extracts by the acid inhibitors of segments 7 to 10, which are almost absent from the root saps. Four promoting zones can be discerned, in segments 1 to 3, 4 to 6, 7 to 8 and 9 to 10. The first three of these are active in both species, but the last only in *E. grandis*. In making species comparisons from these data, it should be noted that the *E. regnans* saps bioassayed were collected from the oldest seedlings available, that is after 30 weeks temperature treatment, while *E. grandis* saps were from the 16 week harvest as usual. Although the seedlings were thus of different ages, they were at roughly the same stage of development in terms of the number of leaf pairs differentiated on the stem.

As in the leaf acid fractions, the promotion in segments 1 to 3 is present in both species at all temperatures. The second zone is also active at all temperatures in *E. grandis* saps, but is not active in *E. regnans* at $30^{\circ}/25^{\circ}$C, in spite of consistent activity in saps from the lower temperature regimes. This corresponds to the segment 5-6 promoter of *E. regnans* leaf acid fractions, which was present at all temperatures at 16 weeks but absent from $30^{\circ}/25^{\circ}$C at 20 weeks. Whether the same compound is involved in activity in this zone in both leaves and root saps, or in both species, is questionable as *E. grandis* leaf acid fractions showed a peak of activity in segments 4 and 5 rather than 5 and 6.

Table 6-10. Results of the cress seed germination bioassay for growth-inhibiting substances in root saps. For explanation of symbols see Table 6-3.

Seedling Source	Rf Segment 1	2	3	4	5	6	7	8	9	10
R30/18	++									
R30/21									++	
R30/24							++		+	
R30/30					+					
G16/18							+			
G16/21	+									
G16/24	+							+		
G16/30			+							
G16/33				++			+			

The segment 7-8 promoter is also absent from *E. regnans* saps at higher temperatures; this may be true of *E. grandis* as well, if the promotion in segment 7 at $33^{\circ}/28^{\circ}C$ is caused by overlapping promoters from the segment 5-6 zone. The segment 9-10 promoter is wholly absent from saps of *E. regnans*, but present over the whole range of temperatures tested in *E. grandis*.

6.3.7. Summary of the bioassay results

The major effects of species, temperature and seedling age on growth substance concentrations detailed above may be summarised as follows:-

1. Acid promoters. Leaf extracts show considerable activity in three zones. The Rf 0-0.3 zone is present at all temperatures and strongest at $24^{\circ}/19^{\circ}$C in both species at 16 weeks, but a reduction with age at this temperature appears to occur in *E. regnans*. The Rf 0.3-0.5 zone is active only in *E. grandis*; it appears to be stronger at $24^{\circ}/19^{\circ}$C and declines in activity with increasing age. The Rf 0.4-0.7 zone, active only in *E. regnans* extracts, is strong after 16 weeks at $30^{\circ}/25^{\circ}$C but absent after 20 weeks at this temperature, correlating with the decline in growth rate in the same period. Root extracts showed relatively weak and sporadic promotion.

2. Acid inhibitors. Rf segments 1 to 4 contained only weak inhibition, mostly from *E. grandis* at low temperatures. Segments 5 and 6 were active only at low temperatures in *E. grandis*, but in *E. regnans* at $30^{\circ}/25^{\circ}$C showed weak activity at 16 weeks, increasing considerably by 20 weeks; this again correlates with the effects of high temperature on the growth of *E. regnans*. Rf segments 7 to 10 were strongly inhibitory at all temperatures in both species. Root extracts again showed less activity than leaf extracts; a species difference was apparent in the absence of inhibition from segments 7 and 8 in *E. grandis* only.

3. Neutral inhibitors. Activity is mainly confined to segments 7-10 in *E. regnans* and 9-10 in *E. grandis*, without observable temperature differences. Segments 7-8 are also active in *E. grandis* in younger material. In root extracts, inhibition is weaker in segments 7 to 10, but stronger in

1 to 6, at least in *E. grandis*. No definite effects of temperature can be found.

4. Aqueous inhibitors. Activity is strongest in Rf segments 6 and 7 of the material examined (*E. grandis* at 10 weeks and *E. regnans* at 20 weeks) and there was no evidence of a temperature effect. Segment 3 is also inhibitory at the highest temperatures tested in both species, and segment 9 shows some inhibition in *E. grandis*.

5. Cytokinins. Bioassay results were variable, but a large species difference is apparent, particularly in the leaves, and temperature effects also seem likely to be present. However, no species - temperature effect was observed.

6. Root sap promoters and inhibitors. Very little inhibition was present in the root saps, but promoting activity exceeded even that of the leaf acid fractions, possibly due to the absence of masking by competing inhibitory compounds. Activity in segments 4 to 6 is present in both species but absent from *E. regnans* at $30^{\circ}/25^{\circ}$C, as in the leaf acid fractions at 20 weeks. Segments 9-10 are active in *E. grandis* only, and segments 7-8 at low temperatures only in both species.

6.4. Discussion

The results of bioassays described above indicate that a large number of active substances with different bio-

logical and chemical properties are present in both species especially in the leaves. There are a number of instances of a substance active in one species being almost or completely absent from the other, and of variation in the concentration of a substance within one or both species with temperature and/or age of the seedlings.

The primary aim of this broad investigation of the growth substance contents of the two eucalypts was to locate differences, between species, in the substances present or more particularly in the effects on them of an increase in growing temperature to $30^{\circ}/25^{\circ}$C and above. At least two clear examples of high temperature effects on the growth substances present in *E. regnans* but not those in *E. grandis* have been demonstrated, and the importance of these is emphasised by the fact that such effects are found only in material from the later harvests. A correlation can therefore be inferred between the concentrations of the substances involved and the strain shown by the seedlings in terms of growth rate and the other symptoms discussed in Chapter 2.

The two growth substances of particular interest, one promoting and one inhibiting, were found in the leaf acid fractions. Although some of the other bioassays demonstrated large differences in growth substance concentrations between species (e.g. cytokinins in the leaf extracts), the acid promoters and inhibitors appear to be the most promising subjects for study in more detail, as the effects of temperature and age on these have been repeatedly shown in bioassays of low variability, and a correlation with growth effects can be seen.

Preliminary studies of the acid inhibitors indicated that the paper chromatographic separation method used did not fully separate all the active compounds present, so that active zones detected in bioassays were in fact due to complexes rather than single compounds. The same is very probably true of the promoter assays: because of this effect, and the possibility that the activity of promoters on some chromatograms may be masked by the presence of inhibitors at about the same Rf, further investigation of these compounds will require a more efficient method of separation than paper chromatography. A more detailed study of the effects of seedling age on growth substance contents of the two species is also required, as only two harvests have been compared in the preliminary assays and no account has been taken of the difference in growth rate between species. The development of suitable techniques for this work is described in Chapter 8, and the bioassay results obtained subsequently are discussed in Chapter 9.

CHAPTER 7

EFFECTS OF TEMPERATURE AND AGE ON AUXIN CONCENTRATIONS IN LEAVES

7.1. Introduction

As discussed in Chapter 5, some of the effects of increasing temperature on the growth and form of *E. regnans* and *E. grandis* seedlings suggest that differences in auxin concentration may exist between seedlings grown at different temperatures, and a similar difference between the two species may be responsible in part for their differing behaviour at $30^{\circ}/25^{\circ}C$ and above. A survey of auxin concentrations in the seedlings over a range of temperatures and ages was therefore needed.

While bioassays of high sensitivity have often been used for the measurement of auxin concentrations in plants, they are time-consuming and may be inaccurate or prone to interference by competing compounds when applied to crude plant extracts (Knegt and Bruinsma 1973). Extensive purification steps including both chromatography and electrophoresis may be required before assay, which again are time-consuming and may also cause appreciable losses of the active substance (Mann and Jaworski 1970). This is also true of some alternative methods used for measurement of auxin concentrations, including gas chromatography (Grunwald *et al.* 1968) and fluorometric assay (Hertel *et al.* 1969).

Stoessl and Venis (1970) proposed an assay for indolyl-3-acetic acid (IAA) in plant extracts which makes use

of the highly specific reaction of this compound with acetic anhydride to produce indolo-α-pyrone, a compound with distinctive ultraviolet, visible and fluorescence spectra. This assay was combined with a rapid extraction method by Knegt and Bruinsma (1973) to provide a procedure for the accurate quantitative determination of nanogram quantities of IAA in crude extracts of plant material.

A number of compounds other than IAA are known to have auxin activity, at least in the straight-growth assay (Galston and Hand 1949), but all the naturally-occurring auxins have been shown to be indole compounds, e.g. indoleacetaldehyde, indolepyruvic acid and indoleacetonitrile, whose auxin activity can probably be accounted for as a result of conversion to IAA (Leopold and Kriedemann 1975). Thus the use of an assay specific for IAA as a measure of auxin activity is justified and may in fact be a better method of determination than less specific assays which include activity due to IAA formed from other indoles during the assay as well as that due to native IAA from the plant. In this regard it is interesting to note that the original bioassay for auxin, the *Avena* coleoptile curvature test of Went and Thimann (1937), is also almost specific for IAA (Leopold and Kriedemann 1975).

7.2. Materials and methods

Assays for IAA were performed only on leaf material. This was sampled from the collections of leaves made at each harvest of seedlings for fresh weight and leaf area determinations, as described in section 6.2.1. In preliminary experiments, the indolo-α-pyrone fluorescence assay as described by

Stoessl and Venis (1970) was attempted on leaf acid fractions extracted as shown in Figure 6-1, but these assays failed due to the formation of water-insoluble compounds in the reaction mixture. These compounds precipitated when the reaction was stopped with water, so that fluorescence measurements were impossible. The same phenomenon was reported by Eliasson *et al*. (1976) who found that the problem could be overcome by stopping the reaction with 50% aqueous ethanol. This practice however reduced the accuracy of the assay.

The use of an extraction method similar to that of Knegt and Bruinsma (1973) proved sufficient to overcome this difficulty, as the acid fractions extracted in this way are virtually pigment-free and considerably cleaner than those chromatographed for the growth substance bioassays of the previous Chapter. The procedure adopted was as follows (Figure 7-1):-

10 g samples of leaves were homogenised with 70 ml of cold redistilled methanol for two minutes at high speed in a Waring blender, and the homogenates stored overnight at $4^{o}C$. Tissue debris was removed by filtration through Whatman no. 41 paper on a Buchner funnel, and washed with further redistilled methanol until the leachate was pale green in colour. The methanol extract was then divided into two equal parts to enable duplicate assays of each leaf sample, and each part was evaporated to an aqueous residue under vacuum in a rotary evaporator at $35^{o}C$.

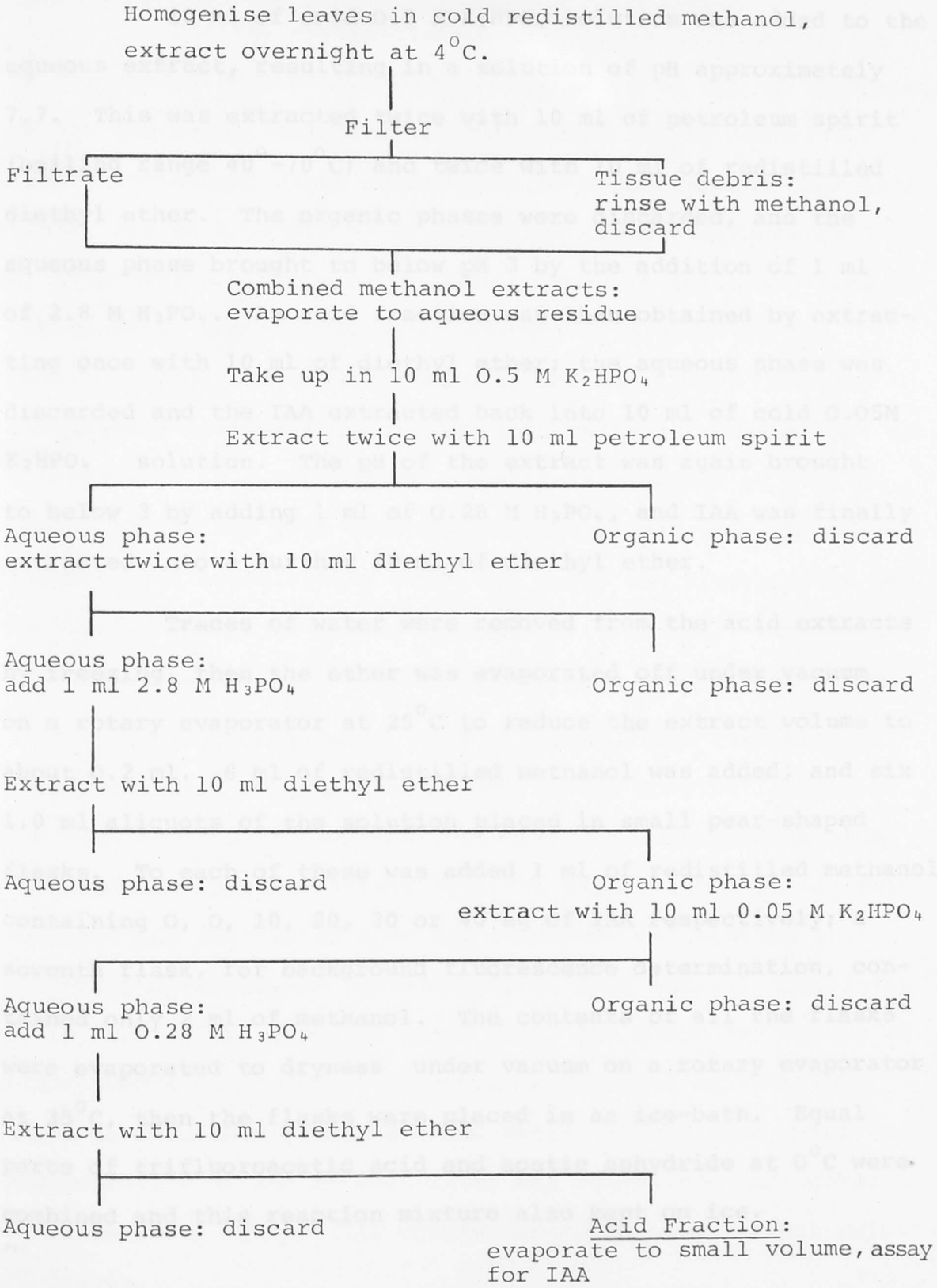


Figure 7-1. Flow diagram illustrating the procedure used for extraction of a purified acid fraction from leaves of *E. regnans* and *E. grandis* prior to assay for IAA.

10 ml of cold 0.5 M K_2HPO_4 solution was added to the aqueous extract, resulting in a solution of pH approximately 7.7. This was extracted twice with 10 ml of petroleum spirit (boiling range 40°-70°C) and twice with 10 ml of redistilled diethyl ether. The organic phases were discarded, and the aqueous phase brought to below pH 3 by the addition of 1 ml of 2.8 M H_3PO_4. An acid fraction was then obtained by extracting once with 10 ml of diethyl ether; the aqueous phase was discarded and the IAA extracted back into 10 ml of cold 0.05M K_2HPO_4 solution. The pH of the extract was again brought to below 3 by adding 1 ml of 0.28 M H_3PO_4, and IAA was finally extracted into a further 10 ml of diethyl ether.

Traces of water were removed from the acid extracts by freezing, then the ether was evaporated off under vacuum on a rotary evaporator at 25°C to reduce the extract volume to about 0.2 ml. 6 ml of redistilled methanol was added, and six 1.0 ml aliquots of the solution placed in small pear-shaped flasks. To each of these was added 1 ml of redistilled methanol containing 0, 0, 10, 20, 30 or 40 ng of IAA respectively; a seventh flask, for background fluorescence determination, contained only 2 ml of methanol. The contents of all the flasks were evaporated to dryness under vacuum on a rotary evaporator at 35°C, then the flasks were placed in an ice-bath. Equal parts of trifluoroacetic acid and acetic anhydride at 0°C were combined and this reaction mixture also kept on ice.

For the spectrofluorometric assay, 0.20 ml of the freshly-prepared reaction mixture was added to six of the flasks in the ice-bath, including the background flask; one of the flasks containing plant extract but no added IAA was used as a blank, and to this 3.0 ml of water was added, followed by 0.2 ml of reaction mixture after 15 minutes. In the other flasks the reaction was stopped after exactly 15 minutes by the addition of 3.0 ml of water at $0^{o}C$, and the flasks contents immediately transferred to a quartz cuvette for measurement of fluorescence in a Farrand Mk 1 spectrofluorometer at a wavelength of 490 nm, with excitation light at 440 nm. The wavelengths used are slightly above and below the fluorescence and excitation maxima of indolo-α-pyrone (Figure 7-2), in order to avoid the possibility of interference from the scatter peaks.

The contents of the background flask were assayed first, and used to establish a zero on the spectrofluorometer. The fluorescence of the blank, resulting from compounds other than indolo-α-pyrone derived from the plant material, was then measured and subtracted from the fluorescence readings subsequently obtained for the aliquots of leaf extract containing 0, 10, 20, 30 and 40 ng of added IAA. The corrected values of fluorescence were used to construct a quenched calibration curve as described by Stoessl and Venis (1970), from which the concentration of IAA in each flask was calculated and converted to an estimate of free IAA concentration in $ng.g^{-1}$ fresh weight of leaves.

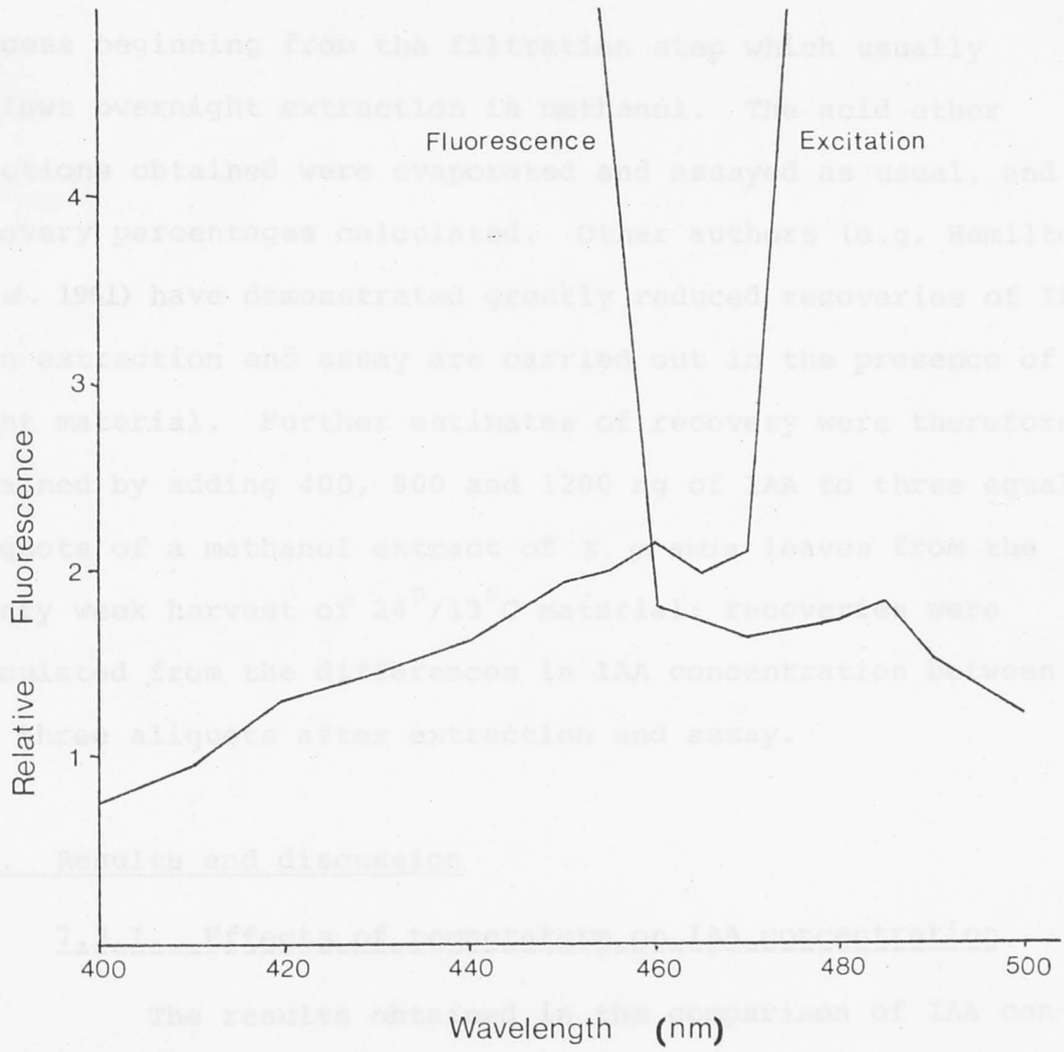


Figure 7-2. Fluorescence and excitation spectra of 40 ng of IAA converted to indolo-α-pyrone. Fluorescence spectrum recorded at 440 nm excitation; excitation spectrum at 490 nm fluorescence.

Estimates of the recovery of IAA resulting from these extraction and assay procedures were obtained both in the presence and absence of plant material. 50 ml of methanol containing 2 µg of IAA was first carried through the extraction process beginning from the filtration step which usually follows overnight extraction in methanol. The acid ether fractions obtained were evaporated and assayed as usual, and recovery percentages calculated. Other authors (e.g. Hamilton *et al.* 1961) have demonstrated greatly reduced recoveries of IAA when extraction and assay are carried out in the presence of plant material. Further estimates of recovery were therefore obtained by adding 400, 800 and 1200 ng of IAA to three equal aliquots of a methanol extract of *E. grandis* leaves from the twenty week harvest of $24^{o}/19^{o}$C material: recoveries were calculated from the differences in IAA concentration between the three aliquots after extraction and assay.

7.3. Results and discussion

7.3.1. Effects of temperature on IAA concentration

The results obtained in the comparison of IAA concentrations in leaves from a range of temperature regimes at the sixteen week harvest are plotted in Figure 7-3. In both species the effect of temperature on leaf auxin concentration is the same, namely a steady increase in concentration with rising temperature over the full range from $18^{o}/13^{o}$C to $33^{o}/28^{o}$C. The two species do differ in the amount of IAA present in their leaves at higher temperatures, concentrations in *E. grandis* being considerably higher at temperatures of $24^{o}/19^{o}$C and above,

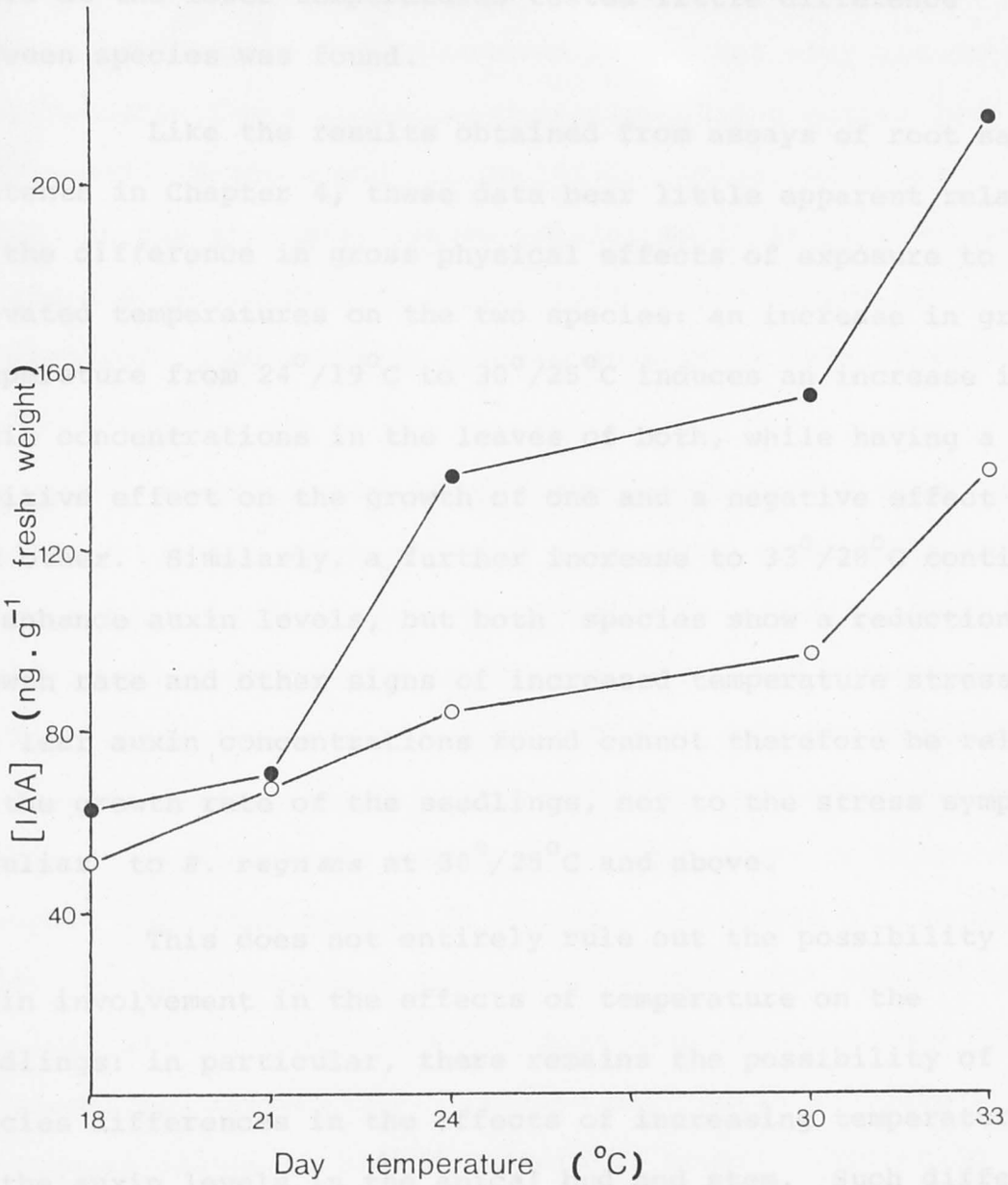


Figure 7-3. Effects of temperature on IAA concentration in leaves of *E. grandis* (●) and *E. regnans* (○). Each point is the mean of two assays.

while at the lower temperatures tested little difference between species was found.

Like the results obtained from assays of root sap contents in Chapter 4, these data bear little apparent relation to the difference in gross physical effects of exposure to elevated temperatures on the two species: an increase in growing temperature from $24^{\circ}/19^{\circ}C$ to $30^{\circ}/25^{\circ}C$ induces an increase in auxin concentrations in the leaves of both, while having a positive effect on the growth of one and a negative effect on the other. Similarly, a further increase to $33^{\circ}/28^{\circ}C$ continues to enhance auxin levels, but both species show a reduction in growth rate and other signs of increased temperature stress. The leaf auxin concentrations found cannot therefore be related to the growth rate of the seedlings, nor to the stress symptoms peculiar to *E. regnans* at $30^{\circ}/25^{\circ}C$ and above.

This does not entirely rule out the possibility of auxin involvement in the effects of temperature on the seedlings: in particular, there remains the possibility of species differences in the effects of increasing temperature on the auxin levels in the apical bud and stem. Such differences could be invoked to account at least in part for the effects of temperature on height growth, stem internode length, cambial activity and apical dominance as reflected in epicormic shoot development. In each case the observed effects would require a decline in auxin concentration at $30^{\circ}/25^{\circ}C$ in *E. regnans* but not *E. grandis*: if such a decline occurs it is in no way reflected in the leaf auxin concentrations.

The role of auxins in leaves is not yet fully understood (Leopold and Kriedemann 1975), but they are generally produced there in large quantities during active leaf enlargement (Wetmore and Jacobs 1953). While auxin has little effect on the growth of leaves (Miller 1951) it is involved in the abscission process (Myers 1940); leaf auxins may also be involved in correlation effects in more or less remote parts of the plant.

Abscission of lower leaves and branches from the seedlings of this study occurred first from those grown at the highest temperatures, particularly from *E. grandis*. This effect could possibly be related to the increased leaf auxin content at high temperatures and generally higher concentrations in *E. grandis* leaves; however, abscission is most often associated with a decreasing auxin supply in an aging leaf or other organ (Leopold and Kriedemann 1975) leading to release of its inhibiting effect on the abscission process, although auxin applied to the proximal side of the abscission zone also promotes this process (Addicott and Lynch 1951). Perhaps high auxin concentrations in younger leaves at high temperatures promote abscission of older leaves lower on the same branch; in any case a correlation appears to exist here between overall leaf auxin levels and the rate of leaf abscission.

7.3.2. Changes in IAA concentration with age

The data of Table 7-1 provide an indication of the variation in auxin content with seedling age at $30^{o}/25^{o}C$, with

some supplementary data from 24°/19°C material. A steady increase in auxin concentration with increasing age was found in both species over the range of ages studied, i.e. up to 16 weeks in *E. grandis* and 20 weeks in *E. regnans*. It is probable that the leaf auxin concentrations approach a plateau after some time, and the values obtained for *E. regnans* at 24°/19°C may illustrate this phenomenon.

Table 7-1. Changes in leaf auxin concentration of *E. regnans* and *E. grandis* seedlings with age, at 24°/19°C and 30°/25°C. Each entry is the mean of two assays, unless otherwise noted.
Units: $ng.g^{-1}$ fresh weight.

Weeks from beginning of experiment	8	10	12	16	20	30
24°/19°C:						
E. regnans				84.5	56.7	83.5
E. grandis				135.7	230.9	
30°/25°C:						
E. regnans	68.8		72.7*	97.2	113.3	
E. grandis	49.7*	62.4	139.7	153.3		

* result of one assay only

As in the temperature data of Figure 7-3 auxin concentrations in *E. grandis* leaves rise more rapidly with time than in *E. regnans* and reach much higher levels, although in young material there is little difference between the two species.

Once again, in seeking a difference in auxin content which correlates with the difference in effects of elevated temperature on growth of the seedlings the 16-20 week period in *E. regnans* at $30^{\circ}/25^{\circ}$C is of particular importance. In the effects of age, as in those of temperature, no change in auxin concentrations occurs in this material which distinguishes it from *E. grandis*; this further supports the conclusion reached in the previous section, that leaf auxin concentrations are not related to the differing effects of high temperature stress on the two species.

7.3.3. Recovery estimates and comments on the methods used

When aliquots of an IAA solution were extracted and assayed by the procedure of section 7.2, recovery estimates of 75.6% and 74.9% were obtained. These are similar to the recoveries of IAA in the absence of plant material calculated by Hamilton *et al*. (1961) using different extraction and assay methods. Mann and Jaworski (1970) however found it necessary to use elaborate precautions, including red glassware, extraction under nitrogen and evaporation at atmospheric pressure to achieve recoveries of this size, either in the presence or absence of plant material. Obviously losses of the order they experienced did not occur during the extraction and assay procedures used here; it is noteworthy however that they found IAA losses during the homogenisation and overnight extraction steps, which were not included in the recovery determinations of this study.

Knegt and Bruinsma (1973) recommended the addition of a small quantity of ^{14}C-IAA to the methanol extract before

purification in order to directly monitor losses for each assay, and reported recoveries of up to 96% obtained in this way in the presence of plant material without resorting to the precautions of Mann and Jaworski (1970). They too added IAA for recovery determination to the methanol extract rather than the original plant material, but as the extraction time used in their original procedure was reduced to 5 to 10 minutes, significant loss of IAA was unlikely to occur.

Eliasson *et al.* (1976) have pointed out that variations in the contents of acid ether extracts from different species may affect their amenability to either bioassays or chemical assays as used here, and by way of example have demonstrated the inability of Knegt and Bruinsma's extraction procedure to produce an extract from *Phaseolus vulgaris* seeds of sufficient purity for assay by the indolo-α-pyrone fluorescence method. It would appear that such differences between species also affect the recovery of IAA attainable in the presence of plant material: the recoveries calculated from assay of two aliquots of IAA in the presence of *E. grandis* leaf extracts were 20.3 and 21.3%, which also are similar to recovery estimates determined by Hamilton *et al.* (1961) for IAA extracted in the presence of etiolated corn shoots.

It appears that eucalypt leaves and corn shoots, at least, contain a component capable of binding IAA, which is not present in the young tobacco leaves, *Begonia* flowers or *Cleome* fruits assayed by Knegt and Bruinsma (1973). Other studies of IAA recovery in the presence of plant extracts have generally used large amounts of added IAA: e.g. Mann and Jaworski (1970) added 10-100 µg of IAA to 30-70 g of soybean

leaves, and Ueda and Bandurski (1969) added 400 µg of IAA to 15 g of ground maize. Recoveries were 44-62% and 65-79% respectively.

Mann and Jaworski (1970), comparing the results of Hamilton *et al.* (1961) with those of other workers, suggested that low levels of IAA may be more readily lost than were high levels. This is compatible with the conclusion (Hamilton *et al.* 1961) that endogenous and exogenous free IAA is bound to some plant component during the extraction process: if the binding substrate is saturated by relatively small quantities of added IAA (of the order of 10 $ng.g^{-1}$ say), very low recoveries will result from the addition of small quantities of IAA (as used here and by Hamilton *et al.* (1961), but also by Knegt and Bruinsma (1973)), while more acceptable recoveries will be obtained from the addition of larger quantities (of the order of 100 $ng.g^{-1}$ or more, as used by Mann and Jaworski (1970), Ueda and Bandurski (1969) and others).

The IAA concentrations determined in this study (Figure 7-3 and Table 7-1) are however quite high, and if the recovery of endogenous free IAA is as low as calculated, the corrected concentrations would be very high. This raises the question of whether exogenous IAA behaves in the same way as the endogenous compound: Hamilton *et al.* (1961) recognised the possibility that it does not, in suggesting their second conclusion that all IAA *in vivo* occurs as a very labile complex, to which the binding of exogenous IAA may or may not be related. If binding of exogenous IAA to a substrate from which endogenous IAA is protected does occur to some extent, then recovery estimates

obtained with saturating levels of added IAA, which approach estimates made in the absence of plant material, will provide a more accurate measure of the recovery of endogenous IAA. As the interpretation of the recovery estimates obtained is thus uncertain, the data of Figure 7-3 and Table 7-1 have been left uncorrected.

Eliasson *et al.* (1976) studied the reliability of the indolo-α-pyrone fluorescence assay for IAA determination in extracts of plants known to give purification problems in bioassays for auxin activity. Experience with the eucalypt leaf extracts assayed here suggests that they caused similar difficulties to the extracts of *Sorbus*, *Betula*, *Populus* and *Pinus* used by the above authors. In addition to the problem of precipitation of water-insoluble substances experienced before the extraction procedure of Knegt and Bruinsma (1973) was adopted (section 7.2), impurities in the acid fractions obtained by this method continued to cause difficulties in some extracts. A number of assays had to be repeated, sometimes with extra purification steps incorporated in the extraction procedure, to avoid very high fluorescence readings resulting from light scattering by turbid solutions. The results obtained thus support the conclusion of Eliasson *et al.* (1976) that the method of Knegt and Bruinsma (1973) is indeed rapid and accurate but demands care and with some plant material may require extra purification of the acid fraction to avoid obtaining misleading results.

CHAPTER 8

ACID INHIBITORS AND GIBBERELLIN-LIKE PROMOTERS : MATERIALS AND METHODS

8.1. Introduction

The preliminary bioassays of leaf and root material described in Chapter 6 demonstrated variations in promoting and inhibiting substances present in the acid fractions of leaf extracts corresponding to variations in the growth and condition of the two eucalypt species at different ages and temperatures. To determine whether the differences observed were due to the presence of different growth substances in the two species or to differences in concentrations of the same substances it was necessary to use more efficient methods of separation than paper chromatography. Improved separation should also reduce the incidence of both promoting and inhibiting compounds occurring in the same bioassay fraction, when one may mask the biological activity of the other.

The promoting and inhibiting activity of the leaf extracts changes with age of the seedling as well as with temperature in both species. The age effect is important in attempting to relate changes in growth substances to the observed decline in growth of *E. regnans* at elevated temperatures after an initial period of healthy vigorous growth. However, the earlier bioassays were based on leaf material sampled at random from whole seedlings without regard to the age of leaf or their stage of development. As it is likely that the growth

substances present change in type and/or amount during the course of leaf development from the bud stage to senescence, a study of the variation in these substances with tree age must employ some form of stratified harvesting to take into account the effects of leaf age. The inclusion of leaves of all ages in the earlier samples could also have led to the partial masking of temperature effects if, for example, these are confined initially to the younger leaves and growing tips of the seedling.

Further deficiencies in the preliminary bioassays stem from the very different rates of development of *E. regnans* and *E. grandis*, and the fact that material was harvested for these bioassays at fixed times after transfer of seedlings to the different temperature regimes, i.e. at 8, 10, 12, 16 and 20 weeks. If the number of stem leaf pairs differentiated is used as an index of development, it can be seen from Figure 2-14 (p. 46) that *E. grandis* seedlings at the 16 week harvest were considerably more mature than *E. regnans*. This may well confound the comparison of the effects of seedling age on growth substance content of the two species. Therefore, harvests should preferably be carried out at equivalent stages of development in each species rather than at definite ages.

Finally, a degree of quantification of the promoters and inhibitors involved is required to facilitate comparisons among species, ages and temperatures. Gibberellin concentrations have been measured spectrofluorimetrically (Elson *et al.* 1964), as well as by gas-liquid chromatography (Cavell *et al.* 1967) and radiochromatography where applicable (Durley *et al.*

1973). However, the gibberellin-like promoters of lettuce hypocotyl elongation found in the leaf acid fractions are not necessarily true gibberellins, and the character of the cress germination inhibitors is wholly unknown. Thus, without isolating and partly identifying each of the active substances, the only practical method of measuring their concentrations in the leaves is by bioassay. While bioassays are subject to inaccuracies due to effects such as masking by competing compounds, they are often the most sensitive methods of detection and measurement of the frequently very low concentrations of growth substances present in extracts of plant material.

For these reasons, another batch of seedlings was grown for assay of growth substances in samples of leaves harvested from different parts of the seedling at definite stages of development. Before routine assays could begin however, some preliminary investigation of the gibberellin-like promoters was desirable. If these showed the characteristic reactions of gibberellins it might be possible to use a fluorimetric assay, at least as a check on bioassays. Partial characterisation of the promoters, if possible, could also be useful in enabling comparisons of the results with those of other workers. A satisfactory method of purification of the crude acid fraction was also required to separate the individual promoters and inhibitors for bioassay without removing any of the substances of interest.

8.2. Preliminary experiments with thin layer chromatography

MacMillan and Suter (1963) described the separation of mixtures of gibberellins and their detection and partial identification by thin layer chromatography (TLC). These techniques were applied to investigate some of the promoting zones located on paper chromatograms of root saps and leaf acid fractions by the bioassays described in Chapter 6. The methods of extraction and initial chromatography have already been detailed in Chapter 6.

Two main zones of promotion were found in the earlier bioassays: one at Rf 0-0.2 and one at Rf 0.5-0.6. The latter zone was apparently more affected by temperature and aging in *E. regnans*. This zone (Rf 0.5-0.6) was eluted with 80% methanol from chromatograms of G16/30 and R16/30 leaf acid fractions, and of G16/33 root sap. The Rf 0-0.2 zone was also eluted from the root sap chromatogram.

The eluates were reduced to a small volume under reduced pressure on a rotary evaporator at 35°C and streaked onto plates of silica gel G. These were then chromatographed using the upper phase of benzene: acetic acid: water (8:3:5) as solvent, after equilibrating overnight with the lower phase. The plates were sprayed with 70% H_2SO_4 in water, allowed to dry, then heated for 10 minutes at 120°C. Examination in ultraviolet light revealed fluorescence as follows:-

Before heating - no fluorescence present.

After heating -

Gl6/33 root sap,	Rf 0-0.2	: fluorescence at Rf	0, 0.8
	Rf 0.5-0.6:	" "	0, 0.2-0.4,0.55
Rl6/30 leaf AF,	Rf 0.5-0.6:	" "	0, 0.3, 0.4
Gl6/30 leaf AF,	Rf 0.5-0.6:	" "	0, 0.2-0.3,0.4

This characteristic formation of fluorescent compounds when heated with sulphuric acid indicates that the promoters of lettuce hypocotyl elongation in the extracts are indeed gibberellins. It is also apparent that the promoting zones from the paper chromatograms each contain more than one gibberellin, and that these can be resolved at least partially by TLC. The Rl6/30 and Gl6/30 eluates may contain the same compounds, and these may also be present in the Gl6/33 root sap if the broad band at Rf 0.2-0.4 was in fact two unresolved bands; this sap also contains another fluorescing compound at Rf 0.55, absent from the leaf acid fractions. Comparison of these results with published data for gibberellin standards (MacMillan and Suter 1963, Cavell et al. 1967) enables a rough characterisation of the gibberellins present: Rf 0 could contain GA1, 2 or 8, or another gibberellin of the same or greater polarity; Rf 0.2-0.3 could contain GA6; Rf 0.4 could be GA5; Rf 0.8 could be GA4. These results are far too tentative to be used for identification, but may indicate the presence of gibberellins of similar chromatographic behaviour and hence the same or similar chemical structure to the known compounds.

As the Gl6/33 root sap appears to contain all the gibberellin-like substances found in the leaf acid fractions,

it was used as a basis for further study and an attempt to correlate fluorescence with biological activity. 6 ml of sap was partitioned three times with n-hexane at pH 7 to obtain a neutral fraction, then three times with diethyl ether at pH 3 to provide an acid fraction. The remaining aqueous fraction was restored to pH 7. All three fractions plus a further 6 ml aliquot of whole sap were evaporated to dryness under reduced pressure, taken up in methanol, streaked onto silica gel G plates and chromatographed using benzene:acetic acid:water solvent as before. A 2 cm strip at the side of each plate was sprayed with 5% H_2SO_4 in ethanol and heated for ten minutes at 120°C.

Fluorescent yellow bands were present in the whole sap, the acid fraction and to a lesser extent the neutral fraction at Rf's 0.25-0.35, 0.35-0.45, and 1.0. The absence of fluorescence at Rf 0, 0.6, and 0.8 as seen previously may be due to insufficient heating with the less severe ethanolic spray. Different gibberellins vary in the period of heating needed to form fluorophors, to such an extent that this property may aid in their identification (Jones *et al*. 1963). The weaker fluorescent bands in the neutral fraction, coinciding with those of the acid fraction, probably indicate that the partitioning method used is not completely efficient. No fluorescent bands were seen in the chromatographed aqueous fraction. The presence of the major part of the fluorescence in the acid fraction further suggests that the substances at about Rf 0.3 and 0.4 are the same as those found in the leaf acid fractions.

The unsprayed parts of the acid fraction and whole sap plates were each eluted in ten equal Rf segments with 4 ml ethanol. The acid fraction eluates were bioassayed in Petri dishes containing 10 lettuce seedlings, as described in Chapter 6. The whole sap eluates were used for fluorescence assay. 4 ml of concentrated H_2SO_4 was added to each eluate after which they were heated in a water bath at $100^{o}C$ for 25 minutes and allowed to cool before measurement of fluorescence in a Farrand Mk 1 spectrofluorometer at a wavelength of 465 nm, with excitation at 417 nm.

Marked fluorescence was shown by the eluates of Rf 0.2-0.3, 0.3-0.4, 0.4-0.5, 0.6-0.7, 0.7-0.8 and 0.9-1.0. These correspond well with the bands observed after spraying the plates, in this and the previous experiment. The attempt to correlate these findings with bioassay activity failed however, as most of the eluates strongly inhibited lettuce hypocotyl growth, probably due to acetic acid remaining on the silica gel when the plate was dried. The inhibition was absent only at Rf 0.9-1.0, where a promotion of hypocotyl elongation equivalent to about 10^{-2} µg GA_3 was observed.

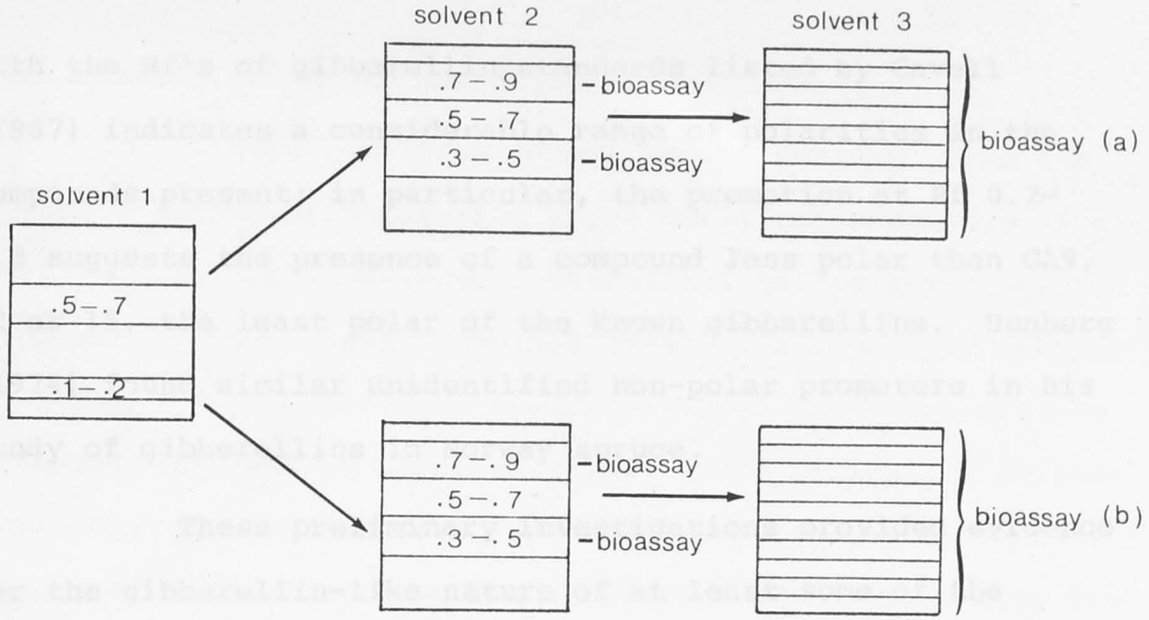
The Rf 0.5-0.6 zones from paper chromatograms of root saps from the R30/24 and R30/30 harvests were similarly examined by TLC and 5% H_2SO_4 spray, as earlier bioassays had shown promotion in the sap from the R30/24 harvest but not from the R30/30 harvest. Several fluorescent bands on the plates before and after spraying made interpretation of the results difficult, but on the R30/24 plate a distinct band appeared after heating at Rf 0.25; this was absent on the R30/30 plate. This band may correspond to the compound active in bioassays,

whose disappearance from the leaves and root saps coincides with the decline in growth of *E. regnans* at $30^{o}/25^{o}$C.

As the previous attempt to locate gibberellin-like substances on thin layer chromatograms by bioassay had failed due to solvent problems, a second attempt was needed to check that the H_2SO_4 spray was indeed detecting active promoters. 5 ml of G16/33 root sap was reduced to a small volume under reduced pressure, streaked onto silica gel G and chromatographed in isopropanol:25% ammonia:water (8:1:1) as used for paper chromatography. A 2 cm strip at the side of the plate was sprayed with 5% H_2SO_4 and heated as usual, while the remainder was eluted from ten equal Rf zones and bioassayed.

The bioassay showed promotion only at Rf 0-0.1 and 0.6-0.7, while three fluorescent bands were detected, at Rf 0.1 (strong), 0.45 (weak), and 0.6 (very weak). To gain further information on the chromatographic behaviour of the compounds and to determine whether the active zones contained one or several promoters, another aliquot of G16/33 sap was chromatographed sequentially in isopropanol:ammonia:water (8:1:1), ethyl acetate:chloroform:acetic acid (15:5:1), and water, then bioassayed as shown in Figure 8-1. Only the middle part of the second plate was eluted for chromatography in water on the assumption that the compounds detected earlier on benzene: acetic acid:water plates would continue to approximate the chromatographic behaviour of GA4, 5 and 6 in ethyl acetate: chloroform:acetic acid.

The bioassay results in Figure 8-1 indicate the presence of several gibberellin-like substances in each active zone of the original chromatogram. Comparison of these results



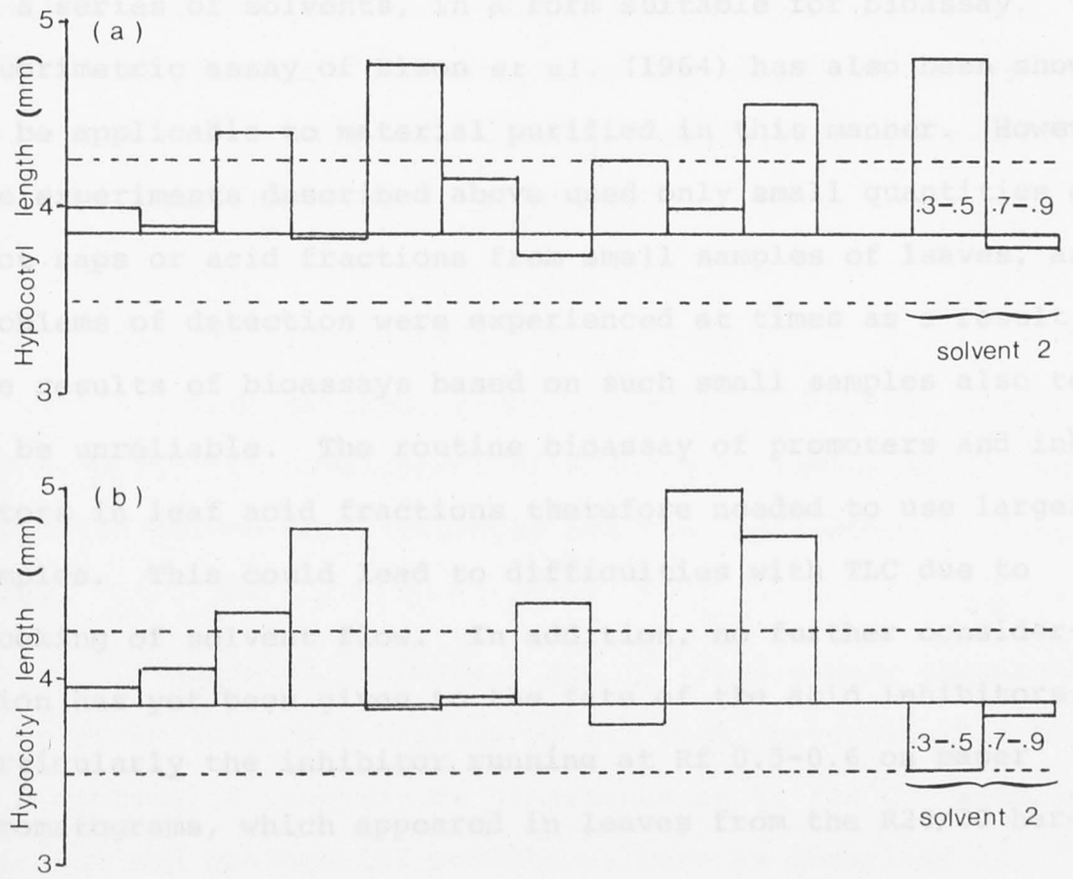


Figure 8-1. Three-stage thin layer chromatography and bioassay of G16/33 root sap.
Solvents: 1-isopropanol:ammonia:water 8:1:1;
2-ethyl acetate:chloroform:acetic acid 15:5:1; 3-water.

with the Rf's of gibberellin standards listed by Cavell (1967) indicates a considerable range of polarities in the compounds present; in particular, the promotion at Rf 0.2-0.3 suggests the presence of a compound less polar than GA9, 12 or 15, the least polar of the known gibberellins. Dunberg (1974) found similar unidentified non-polar promoters in his study of gibberellins in Norway spruce.

These preliminary investigations provided evidence for the gibberellin-like nature of at least some of the promoting substances detected in bioassays, and demonstrated that these substances can be separated successfully by TLC in a series of solvents, in a form suitable for bioassay. The fluorimetric assay of Elson *et al.* (1964) has also been shown to be applicable to material purified in this manner. However, the experiments described above used only small quantities of root saps or acid fractions from small samples of leaves, and problems of detection were experienced at times as a result; the results of bioassays based on such small samples also tend to be unreliable. The routine bioassay of promoters and inhibitors in leaf acid fractions therefore needed to use larger samples. This could lead to difficulties with TLC due to blocking of solvent flow. In addition, no further consideration has yet been given to the fate of the acid inhibitors, particularly the inhibitor running at Rf 0.5-0.6 on paper chromatograms, which appeared in leaves from the R20/30 harvest.

8.3. Liquid partition column chromatography

Liquid partition column chromatography is an alternative method of separation which could overcome some of the difficulties outlined above. The silica gel partition method proposed by Powell and Tautvydas (1967) and developed further by Durley *et al.* (1972) is frequently used in the separation of gibberellins from plant material. Such silica gel columns reputedly give better resolution and more repeatable results than are obtainable from TLC (Durley *et al.* 1972). They are also adaptable to larger extracts and are simpler and more reliable than a multi-step TLC process in which losses of active compounds may occur during elution from each plate. Silica gel partition chromatography was therefore tested as an appropriate method for routine purification and separation of plant extracts prior to bioassay.

8.3.1. Plant material

Plant material grown and extracted as set out below was used for the development of a suitable technique of liquid partition column chromatography and its application to routine assay.

Seeds of *E. regnans* and *E. grandis* were germinated in trays containing a mixture of 1:1 perlite-vermiculite held in a controlled temperature glasshouse at 24°/19°C. Watering, nutrient and photoperiod conditions were as described in Chapter 2. When the seedlings had developed two pairs of leaves they were transplanted into pots containing 1:1 perlite-

vermiculite, two seedlings to a pot, and were kept at $24^{o}/19^{o}C$ for a further two weeks. Half the pots were then transferred to a glasshouse at $30^{o}/25^{o}C$. At this stage the *E. regnans* seedlings had three leaf pairs while those of *E. grandis* had four.

Only two temperature regimes were included to simplify the study of temperature-age interactions and keep the experiment to a manageable size while allowing a detailed study of the effects of leaf and plant age on growth substance concentrations. At $30^{o}/25^{o}C$ the differences between the species and the effect of age on growth of *E. regnans* were both well displayed, while $24^{o}/19^{o}C$ provided a satisfactory reference temperature at which both species were expected to remain healthy and vigorous for the duration of the experiment.

On reaching the 6 leaf-pair stage one seedling from each pot was discarded to leave an essentially uniform population of 15 seedlings per species - temperature group. Each of the seedlings was numbered and assigned to one of three sets, for harvest at the 12, 18 or 24 leaf-pair stage respectively. The relation between development in terms of number of stem leaf-pairs differentiated and chronological age for each species-temperature group is shown in Figure 8-2. The temperature/age effect and the difference between species as previously shown in the growth study (Chapter 2) were repeated here.

The number of branches at each node and the number of leaves on each branch were recorded for every tree at the 6 leaf-pair stage. At the 12 leaf-pair (12L) harvest it was then possible to distinguish the leaves formed during development of the first 6 stem internodes (the 0-6 leaves) from those

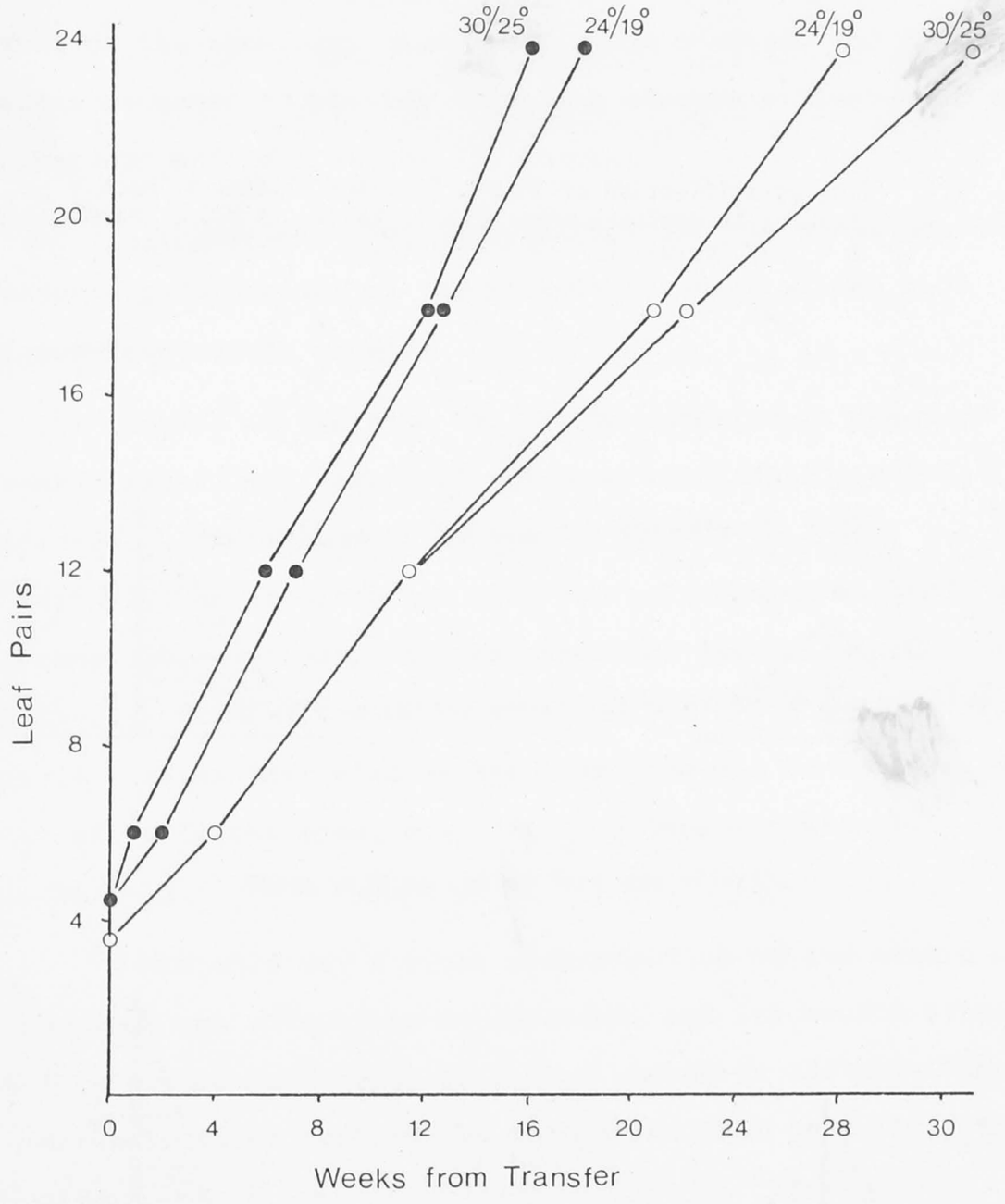
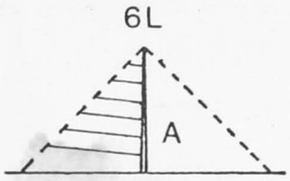
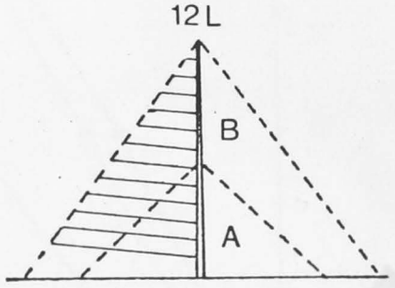
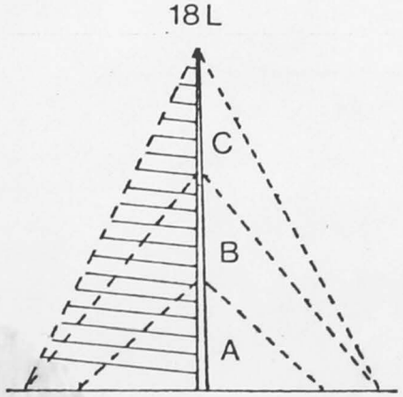


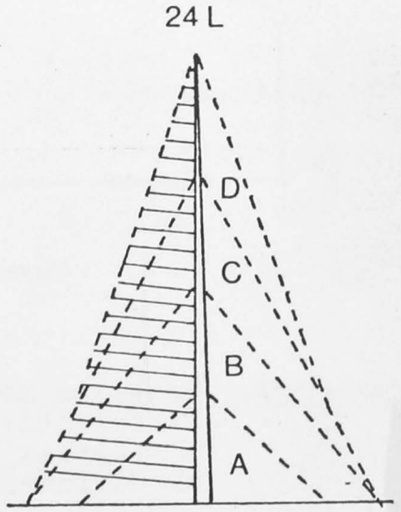
Figure 8-2. Relation between number of stem leaf pairs differentiated and time since transfer of *E. regnans* (○) and *E. grandis* (●) seedlings to different temperature regimes.

[1]The stratification of leaves into age classes at each harvest is depicted more clearly below. A = 0-6 leaves, B = 7-12 leaves, C = 13-18 leaves, D = 19-24 leaves.









formed since (the 7-12 leaves). All the leaves from five seedlings per group were collected in these two classes, and stored at -20°C until needed for extraction. The branches and leaves on the remaining trees were again counted, and the process repeated to provide three age classes of leaves at the 18L harvest and four at the 24L harvest.[1]

This stratified harvesting technique makes possible a three-way comparison of the effects of aging within each species-temperature group:

(i) leaves of the same age can be compared on trees of different ages, e.g. 12L/7-12 compared with 18L/13-18 and 24L/19-24. These will be called seedling age effects.

(ii) leaves of different ages can be compared on trees of the same age, e.g. 24L/7-12 compared with 24L/13-18 and 24L/19-24. These will be called effects of position in the seedling.

(iii) leaves initiated at the same time can be compared on trees of different ages, e.g. 12L/7-12 compared with 18L/7-12 and 24L/7-12. These will be called leaf age effects.

In this way a clear understanding of the nature of an apparent age effect can be obtained, and its source attributed to either leaf aging or a true change in the physiology of the whole plant such as the transition from juvenility to maturity.

A total of nine samples of leaves was collected from each species-temperature group, but the 0-6 leaf samples were always very small and were not used for bioassay. After preliminary extraction and bioassay experiments there was also insufficient 12L/7-12 material from 30°/25°C to be included in the main assessment of promoters and inhibitors in the

leaves. A total of 22 samples of leaves was in fact bio-assayed.

8.3.2. Extraction of the crude acid fraction

Samples of frozen leaves, usually 25 g fresh weight, were homogenised in cold methanol (5 $ml.g^{-1}$ fresh weight of tissue) in a Waring blender, and allowed to stand overnight at $4^{o}C$. The extract was filtered through Whatman No. 41 filter paper in a Buchner funnel and the tissue debris washed with methanol until the leachate was light green in colour. The methanolic extract was evaporated under vacuum in a rotary evaporator at $35^{o}C$ to an aqueous residue. The residue was washed from the evaporating flask with 0.5 M K_2HPO_4 (1 $ml.g^{-1}$ of tissue), then centrifuged for 20 minutes at 2500 g to remove suspended pigments and any remaining solid matter, leaving a clear supernatant which was coloured brown from *E. grandis* leaves and yellow from *E. regnans*.

The supernatant was adjusted to pH 8.0 with 10 N KOH, then partitioned three times against equal volumes of redistilled diethyl ether to remove "neutral" substances (see Figure 8-3). The use of pH 8 rather than pH 7 as previously will minimise loss of less polar gibberellins into the ether phase, which may be considerable at pH 7 (Durley and Pharis 1972). The ether fraction was discarded, and the buffer fraction acidified to pH 3.0 with 10 N HCl before partitioning four times against equal volumes of redistilled ethyl acetate. The combined ethyl acetate fractions were evaporated to dryness

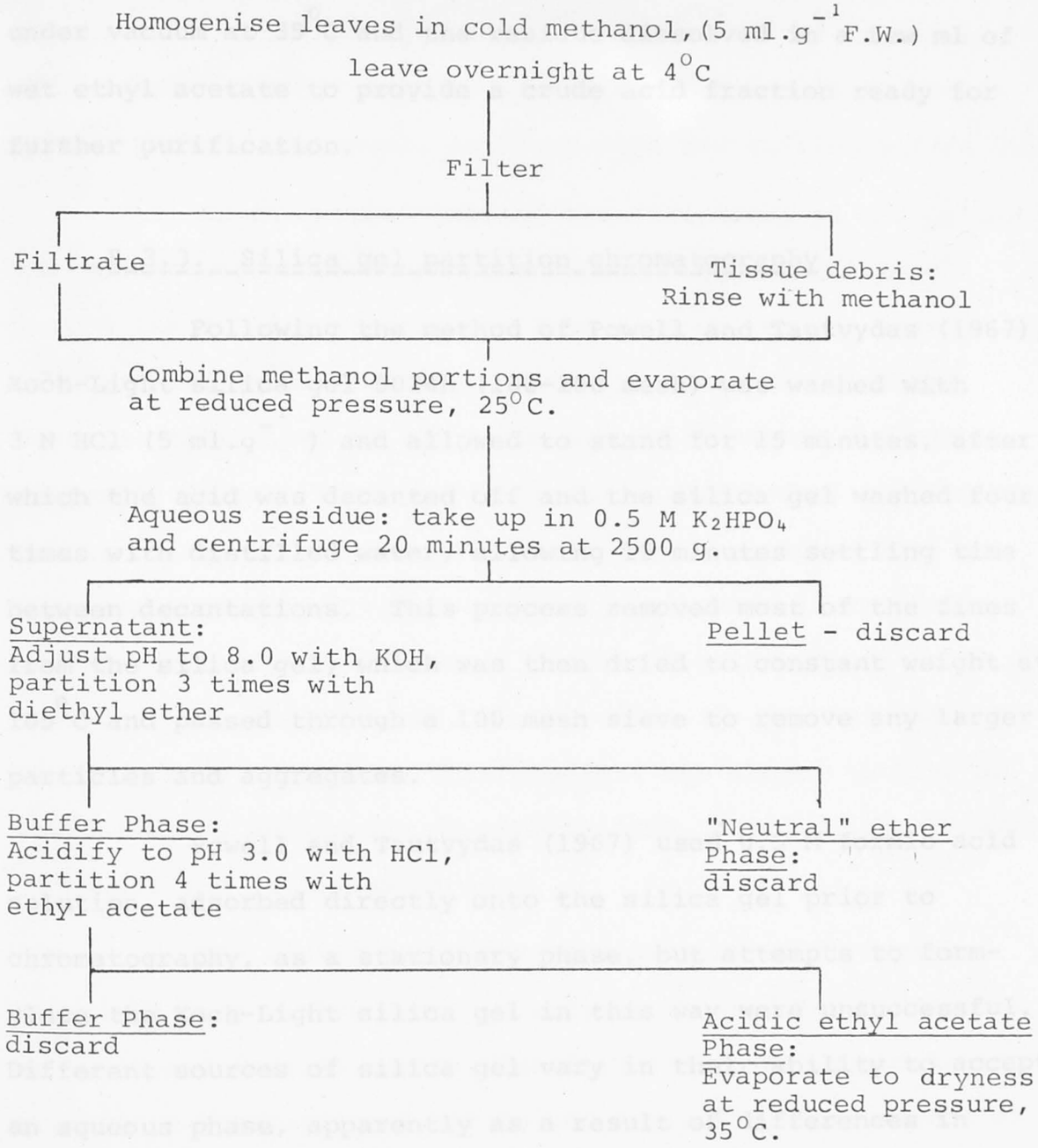


Figure 8-3. Flow diagram showing procedure for extraction of a crude acid fraction from eucalypt leaves for further purification and bioassay.

under vacuum at 35°C and the residue dissolved in a few ml of wet ethyl acetate to provide a crude acid fraction ready for further purification.

8.3.3. Silica gel partition chromatography

Following the method of Powell and Tautvydas (1967), Koch-Light silica gel 5024h (100-200 mesh) was washed with 3 N HCl (5 $ml.g^{-1}$) and allowed to stand for 15 minutes, after which the acid was decanted off and the silica gel washed four times with distilled water, allowing 15 minutes settling time between decantations. This process removed most of the fines from the silica gel, which was then dried to constant weight at 105°C and passed through a 100 mesh sieve to remove any larger particles and aggregates.

Powell and Tautvydas (1967) used 0.5 M formic acid solution, adsorbed directly onto the silica gel prior to chromatography, as a stationary phase, but attempts to formylate the Koch-Light silica gel in this way were unsuccessful. Different sources of silica gel vary in their ability to accept an aqueous phase, apparently as a result of differences in pore size within the particles (Lamontagne and Johnson 1970). Durley *et al.* (1972) compared a number of sources, and in at least one case were also unable to directly adsorb formic acid onto the silica gel but found that this initial adsorption was unnecessary as long as formic acid-saturated solvents were used in eluting the column. 20 g of silica gel was therefore slurried with 40 ml of formic acid-saturated ethyl acetate and washed into a 16 mm internal diameter glass column. The column was packed by pressure from a squeeze bulb to a final

height of 13 cm, then washed with a further 30 ml of formic acid-saturated ethyl acetate and 40 ml of formic acid saturated n-hexane. As usual, solvents were redistilled before use.

As an initial test of the efficiency of the column for separating gibberellin-like substances from eucalypt leaves the dried acid fraction from 25 g of leaves, with 20 µg of GA_3 added, was taken up in a small volume of wet ethyl acetate and applied to two 16 mm discs of Whatman 3MM chromatography paper. These were allowed to dry before placing them gently on top of the column. The column was eluted with a stepwise gradient of ethyl acetate in n-hexane (both formic acid-saturated), consisting of twelve 20 ml fractions containing 0, 10, 15, 17.5, 20, 25,40, 42.5, 45, 70, 100 and 100 per cent ethyl acetate. This gradient was chosen to give an initial broad separation of gibberellins, based on the results of Powell and Tautvydas (1967).

The eluted fractions were evaporated to dryness, dissolved in 4 ml 50% H_2SO_4 in ethanol, heated for 25 minutes at 100°C and assayed spectrofluorimetrically using excitation light at a wavelength of 417 nm and measuring fluorescence at 465 nm as before. Peak fluorescence was found in the 42.5% ethyl acetate fraction: Powell and Tautvydas' results indicated that this fraction should contain GA_3. The adjacent fractions also fluoresced quite strongly, and other fluorescence peaks occurred in the 15% and the second 100% ethyl acetate fractions, with weaker fluorescence at 0%, 10% and 17.5%.

The presence of fluorescence in most of the fractions could be due to poor resolution of a small number of

gibberellin-like substances, or the presence of a larger number spanning a considerable range of polarity. By collecting a greater number of fractions from a gradient of smaller steps it should be possible to separate these substances if resolution is reasonably good. Another column was prepared as before and loaded with the acid fraction from 50 g of leaves on paper discs for elution with sixteen 50 ml fractions in a gradient consisting of 0, 5, 10, 12, 14, 16, 18, 20, 25, 30, 40, 45, 50, 60, 100 and 100 per cent ethyl acetate in n-hexane. Thirty-two 25 ml fractions were collected in order to improve separation; a 5 ml aliquot of each was bioassayed using lettuce seedlings and cress seeds, and the remaining 20 ml subjected to fluorimetric assay as before.

The bioassay results indicated separation of at least four gibberellin-like substances with very good resolution, at 0, 10, 14, and 18% ethyl acetate. Only the first two of these were detected by the fluorescence assay; however, background fluorescence in control solutions was quite strong and could have hidden a weak fluorescence peak from a gibberellin present in low concentration or one which formed a fluorophor of relatively low intensity. Fluorescence assay was deemed unsuitable for subsequent detection and measurement of growth promoting compounds in the leaf material.

Of more concern was the presence of a fluorescence-quenching,strongly inhibiting compound in fractions 17 to 32 inclusive, preventing detection of any promoters which may have been present in these fractions by either assay. This effect was most intense in the 100% ethyl acetate fractions (29 to 32), which were toxic to both lettuce and cress and formed very

dark solutions when heated with ethanolic sulphuric acid. This inhibition was at first thought to be caused by the inhibitor complex located at Rf 0.6-0.9 on paper chromatograms in the bioassays described in Chapter 6. Since it could mask the presence of more polar gibberellin-like substances, a method of separating out this inhibition was sought.

The acid fraction obtained from 50 g of leaves was applied to a column of insoluble polyvinylpyrrolidone (PVP) prepared as described by Glenn *et al.* (1972) and used by Dunberg (1974) to remove inhibitory substances from acid fractions of spruce tissue prior to assay for gibberellins. The column was eluted with 0.1 M phosphate buffer at pH 8, and the 15 to 150 ml eluate collected. Preliminary tests with an identical column showed that GA_3 eluted between 50 and 70 ml. Subsequent silica gel column chromatography and bioassay showed that the inhibition was still present in fractions 17 to 32.

The portions of fractions 17 to 28 and 29 to 32 which had not been used for bioassay were bulked separately, reduced in volume and streaked onto silica gel G plates. After chromatographing with water as solvent the plates were divided into ten equal Rf segments, each of which was eluted with wet ethyl acetate and bioassayed with lettuce seedlings and cress seeds. Inhibition was localised in Rf 0.7-0.9 of the plate carrying fractions 17 to 28, and at least two gibberellin-like promoters were revealed at Rf 0.2-0.3 and 0.5-0.6 on this plate. Although fractions 29 to 32 were strongly toxic to cress seeds and lettuce seedlings when bioassayed directly from the column, the plate carrying fractions 29 to 32 showed only weak inhibi-

tion at Rf 0.9-1.0. The strongly inhibiting material apparently did not elute from the TLC plate.

The failure of the PVP column to remove or reduce the effect of the inhibiting material, combined with its extremely poor resolution on silica gel columns and severe inhibition or toxicity in bioassays together suggested that it was unlikely to be derived from the plant material. To confirm this, a silica gel column was prepared as before and 100 µg GA_3 was applied on a paper disc. The column was eluted with the 16 X 50 ml fraction gradient described earlier and the eluates were bioassayed using cress seed and lettuce seedlings. Inhibition was again found in fractions 17 to 32, but was less intense in fraction 24 (40% ethyl acetate) where GA_3 is expected to elute. It was concluded that the large volume of solvent used for elution with this gradient (800 ml) allowed some of the formic acid present in the solvent to pass through the column rather than be completely adsorbed by it. Formic acid caused the severe inhibition in fractions 17 to 32; this accounts for both the poor resolution of the inhibitor and its peak activity in the last four fractions eluted.

The technique was modified to overcome this problem. The column length was increased by 25% (25 g silica gel instead of 20 g) and a gradient consisting of twenty-five 20 ml fractions containing 0, 4, 6, 8, 10, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 100, and 100 per cent formic acid-saturated ethyl acetate in formic acid-saturated n-hexane (total volume 500 ml) was used. A trial run with GA_3 again showed strong inhibition in fractions 16 to 18 and 20 to 25; GA_3 apparently reduced the inhibition in fractions

19 and 20. Thus in spite of the increased column length and decreased volume of eluant, formic acid is still eluted from the column by ethyl acetate concentrations greater than 25%.

The initial tests with silica gel columns successfully used up to 100% ethyl acetate, while the total volume of eluant was only 240 ml; with the 16 X 50 ml gradient inhibition occurred after 400 ml elution volumne, i.e. at ethyl acetate concentrations of 25% and above. With the new gradient and column size, inhibition occurred after 300 ml elution volume, again corresponding to ethyl acetate concentrations greater than 25%. The elution of formic acid from the column is therefore influenced both by the volume of eluant and its polarity, i.e. ethyl acetate concentration. This effect of concentration occurs as a result of the greater solubility of formic acid in ethyl acetate than in n-hexane. As both solvents are saturated with formic acid, the gradient of ethyl acetate concentration is accompanied by a gradient of formic acid concentration; once the capacity of the column to adsorb formic acid from the eluant is exceeded, it elutes freely in increasing concentration as the ethyl acetate concentration is increased. Powell and Tautvydas (1967), using a column made from 8 g of silica gel and eluted with 800 ml of solvent, apparently did not experience this problem: as mentioned previously, different sources of silica gel vary considerably in their ability to adsorb an aqueous phase, and the Koch-Light silica gel used here obviously has a relatively low capacity for adsorbing formic acid.

To avoid further decreasing the number of fractions collected or their size in order to prevent elution of formic

acid and thereby losing some of the resolving power of the column, it was decided to utilise the earlier finding (p.173) that TLC in water removes the formic acid from the eluate. The following procedure was designed:-

25 g of acid-washed, dried and sieved silica gel was slurried with 40 ml of formic acid-saturated ethyl acetate (prepared by shaking 250 ml redistilled ethyl acetate with 10 ml 0.5 M HCOOH solution and discarding the lower phase) and poured into a 16 mm i.d. glass column. The column was packed by pressure from a squeeze bulb to a height of 17.5 cm, then washed with a further 30 ml of formic acid-saturated ethyl acetate and 40 ml of formic acid-saturated n-hexane (prepared as above but with 5 ml HCOOH solution per 250 ml redistilled n-hexane).

The dried acid fraction from each sample of plant material, extracted as described in the previous section, was taken up in a few ml of wet ethyl acetate and absorbed onto four 16 mm discs of Whatman no. 3MM chromatography paper. These were allowed to dry and placed on top of the column, which was then eluted with a gradient of fifteen 20 ml fractions containing 0, 4, 6, 8, 10, 12, 13, 14, 15, 16, 17, 18, 19, 20 and 25 per cent formic acid-saturated ethyl acetate in n-hexane, i.e. the first fifteen fractions of the 25 X 20 ml fraction gradient used previously. The flow rate of the solvent was maintained at 3-3.5 ml/minute, using a pump where necessary. The column was finally eluted with 80 ml of 70% formic acid-saturated ethyl acetate in n-hexane to remove the more polar compounds. This final eluate was evaporated to dryness under reduced pressure, taken up in a small volume of

wet ethyl acetate and streaked onto a silica gel G plate for TLC in water. After drying, the plate was divided into ten equal Rf segments. The combined column/TLC technique thus separated the crude acid extract into twenty-five fractions for bioassay.

After the extraction and assay of gibberellins and inhibitors from eucalypt leaves by the methods described in this Chapter had been completed, Browning and Saunders (1977) published the results of experiments which suggest that the accepted technique of methanol extraction does not extract certain membrane-bound gibberellins from wheat leaves. If the same is true of eucalypt leaves it could affect the results of this study, so the methods of Browning and Saunders (1977) were applied to the extraction of gibberellins from samples of the material used here and the concentrations obtained were compared with those in methanol extracts of the same material. The details of this work are described in the Appendix.

8.3.4. Bioassays

The lettuce hypocotyl and cress seed bioassays were applied as described in Chapter 6, using 10 lettuce seedlings and 10 cress seeds on filter paper in a 9 cm Petri dish with 5 ml water for each column fraction or TLC segment. Column fractions were simply poured into the dishes and allowed to evaporate, leaving the active compounds on the filter paper. The silica gel scraped from TLC plates was not eluted, but simply placed on the filter paper as preliminary tests showed no effect of silica gel alone on either bioassay. Controls were included with each bioassay. These consisted of either

50% n-hexane:50% ethyl acetate eluted from the column before applying the discs containing the extract, or silica gel scraped from a blank TLC plate run in water, as appropriate.

To allow quantitative estimates of the concentrations of promoters and inhibitors in leaves, dosage-response curves for GA_3 and abscisic acid (a representative acid inhibitor) were obtained by bioassay of a series of standard solutions of these two substances (Figure 8-4). Simple linear regressions were calculated from the data, using a logarithmic transformation for the lettuce bioassay and a probit transformation for the cress bioassay. The regression equations were used to convert significant bioassay responses to an estimate of the equivalent concentration of GA_3 or abscisic acid per kg fresh weight of leaves.

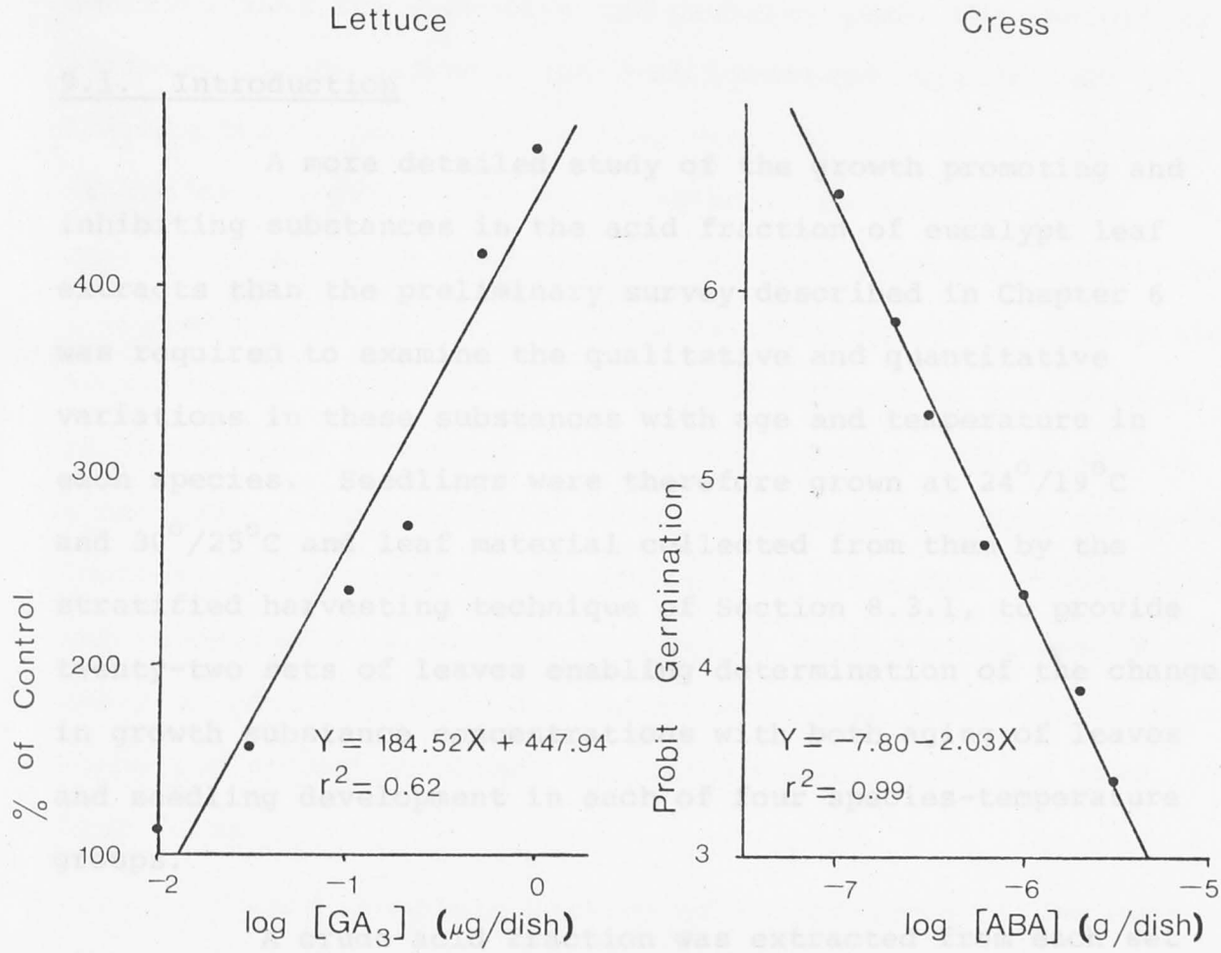


Figure 8-4. Responses of lettuce and cress to standard concentrations of GA_3 and Abscisic acid (ABA) respectively.

CHAPTER 9

EFFECTS OF TEMPERATURE AND AGE ON GIBBERELLIN-LIKE PROMOTERS AND ACID INHIBITORS

9.1. Introduction

A more detailed study of the growth promoting and inhibiting substances in the acid fraction of eucalypt leaf extracts than the preliminary survey described in Chapter 6 was required to examine the qualitative and quantitative variations in these substances with age and temperature in each species. Seedlings were therefore grown at $24^{\circ}/19^{\circ}C$ and $30^{\circ}/25^{\circ}C$ and leaf material collected from them by the stratified harvesting technique of Section 8.3.1, to provide twenty-two sets of leaves enabling determination of the changes in growth substance concentrations with both aging of leaves and seedling development in each of four species-temperature groups.

A crude acid fraction was extracted from each set of leaves and separated by column and thin layer chromatography, using the methods developed in Chapter 8. The twenty-five fractions resulting from this separation process were bioassayed for both promoting and inhibiting activity, using the lettuce hypocotyl elongation and cress seed germination assays. Bioassay responses significantly different from controls at the 5% probability level were expressed as equivalent concentrations of GA_3 or abscisic acid (ABA) by the application of regression equations derived from the dosage-response data of Figure 8-4.

Limitations of both time and quantity of material available made simultaneous assay for both types of substance on the same extracts in this way essential, although it was realised that the promoting and probably also the inhibiting activity of the extracts could be increased considerably by introducing further purification steps geared to the isolation of one or the other type of substance. As a result, the changes in apparent concentration of both promoters and inhibitors described in the following sections should be interpreted as shifts in a balance of interacting compounds; for example, an increase in promoting activity may equally well be due to a decrease in concentration of an inhibiting compound as to an increase in promoter concentration. In bioassays of relatively impure extracts as carried out here to enable simultaneous assay of both promoters and inhibitors, such interactions of competing active compounds are unavoidable. They may take four forms:-

(i) Complete masking of the presence of one compound by the activity of the other. The likelihood of this is reduced by collecting a large number of fractions, as long as resolution remains good. The gradient used for eluting the column in fractions 1-15 is not steep, rendering complete masking unlikely there. The complex of inhibitors in fractions 21-24 from TLC of a number of extracts, and that in fractions 11-15 of the extract from G24L/30 leaves 19-24, may however hide promoters present in the same fractions.

(ii) Partial masking, or diminution of the activity of a substance is almost certain to occur throughout the bioassays. Crozier *et al.* (1969) demonstrated a marked increase in gibberellin bioassay activity in an extract from Douglas fir shoots at each stage of a multi-step purification procedure, and preliminary experiments with a PVP column indicated that similar enhancement of the biological activity of eucalpyt leaf extracts is readily attainable, if only gibberellin-like promoters are to be bioassayed. For the combined bioassays used here, such masking is regarded as acceptable as it does not prevent comparisons of the activity of the same fractions from different extracts; however, the concentrations of promoting compounds in GA_3 equivalent units are almost certainly under-estimated as a result.

(iii) An apparent shift in the position of elution of a compound may result from the modification of its activity by competing compounds in adjacent fractions. The elution of most compounds spreads over more than one fraction, but masking of activity in one of these makes a compound appear to elute only in the other(s). The classification of active fractions in the bioassays into zones of activity likely to result from the same compound is complicated by such effects.

When more than one bioassay is carried out on the same extract, activity due to a particular substance is often located in different fractions depending on the assay used. This can be seen in Dunberg's (1974) bioassays for promoters, and here in a comparison of the cress bioassay results with inhibition in the lettuce bioassay. The fact is that chroma-

tographic separation of plant extracts is seldom clear cut, and each fraction usually contains a number of substances which may or may not interact to produce a bioassay response. The apparent location of an active compound then depends on the sensitivity of the particular species used as bioassay material to these complexes of interacting substances.

(iv) A related effect to such shifts in the apparent position of elution of a compound may occur where resolution is relatively poor and the substance is present in high enough concentration to produce a significant bioassay response in three or more fractions. In this case it is possible for a competing compound to completely mask the response in the middle fraction, causing the substance to appear as two separate peaks. The number of active zones from bioassay data may therefore be greater than the number of compounds producing them.

The opposite effect, of identifying fewer zones than the number of compounds they represent, may of course also occur if the extract contains active substances of similar chromatographic behaviour, which elute in the same or adjacent fractions. Thus only after extensive purification of an active fraction can one realistically attribute activity to single compounds. It is nonetheless convenient for purposes of comparison between plant extracts to classify the bioassay responses into active zones on the basis of repeated occurrence in the same or adjacent fractions, and to compute the relative activities of these zones as if they were in fact discrete compounds.

9.2. Bioassay results

The results of bioassays of twenty-two acid extracts from the leaves of *E. regnans* and *E. grandis* are presented in Figures 9-1 (lettuce) and 9-2 (cress). The location of biological activity in a particular fraction may be assumed reproducible to ± one fraction, both for the TLC and column separations (Durley *et al.* 1972). This amount of variation is to be expected as a result of small differences in running conditions between different columns and TLC plates, and differences in the quantity of gums and other interfering compounds in the crude acid fractions. The apparent location of an active compound may also be affected by the presence of competing substances as discussed above. Allowing for this variation, the fractions with significant biological activity can be classified into eight promoting and six inhibiting zones (see Figures 9-1 and 9-2) with only a few ambiguous bioassay responses.

The bioassay data, converted to equivalent concentrations of GA_3 or ABA in each active zone, are listed in full in Tables 9-1 to 9-4, and discussed in the following sections. Each entry in these Tables is based on bioassay of a single leaf extract, and the results must be interpreted bearing in mind the possible variation between extracts of leaf samples from the same harvest.

9.2.1. Growth promoting substances

9.2.1.1. Effects of leaf age

The effects of aging on the concentrations of gibberellin-like promoters in leaves initiated on seedlings at

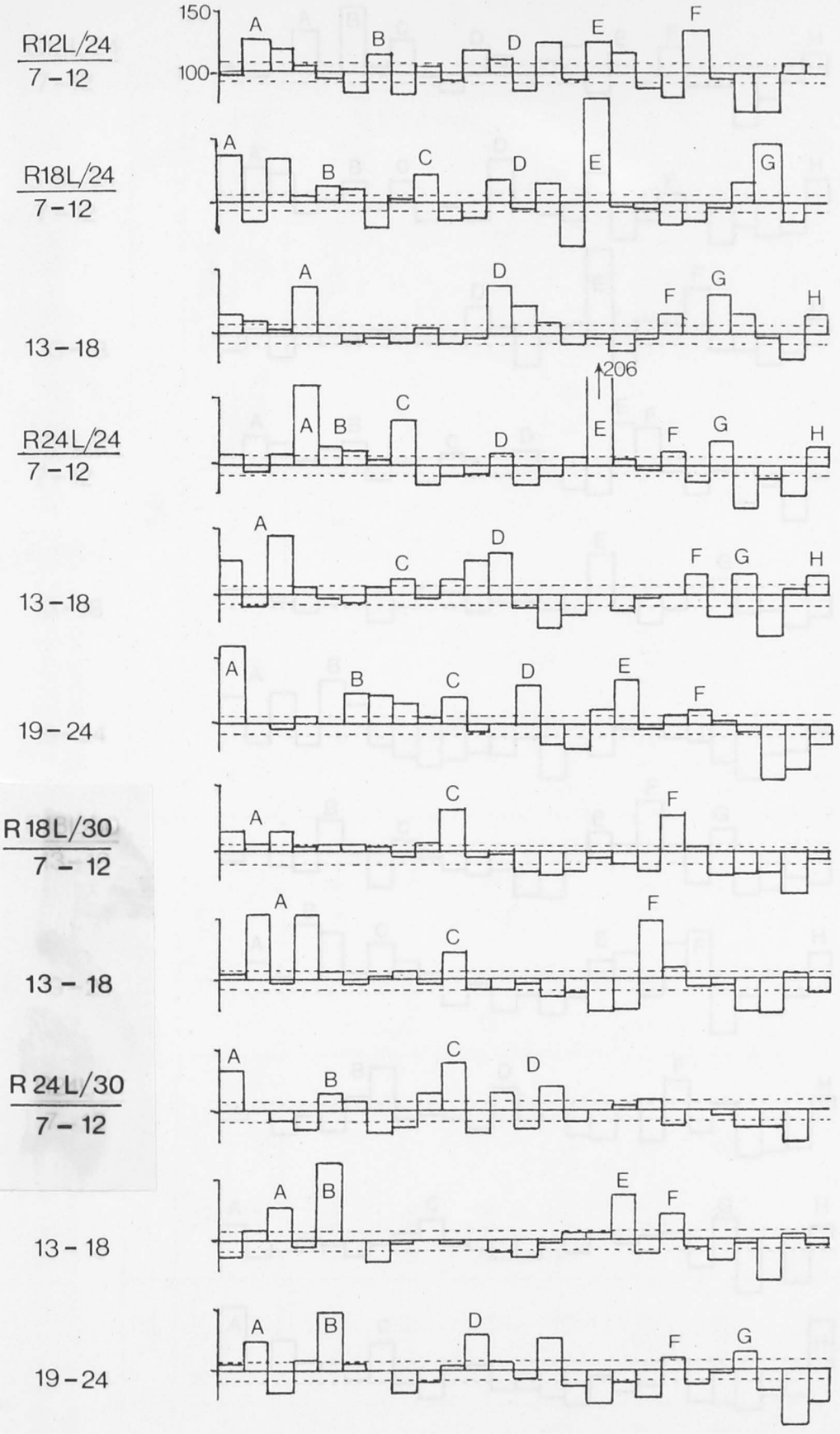


Figure 9-1. Histogrammed results of lettuce bioassays of 22 leaf extracts. Ordinate: percentage of control hypocotyl length. Abscissa: fractions 1-25. Dashed lines indicate 95% confidence limits for controls.

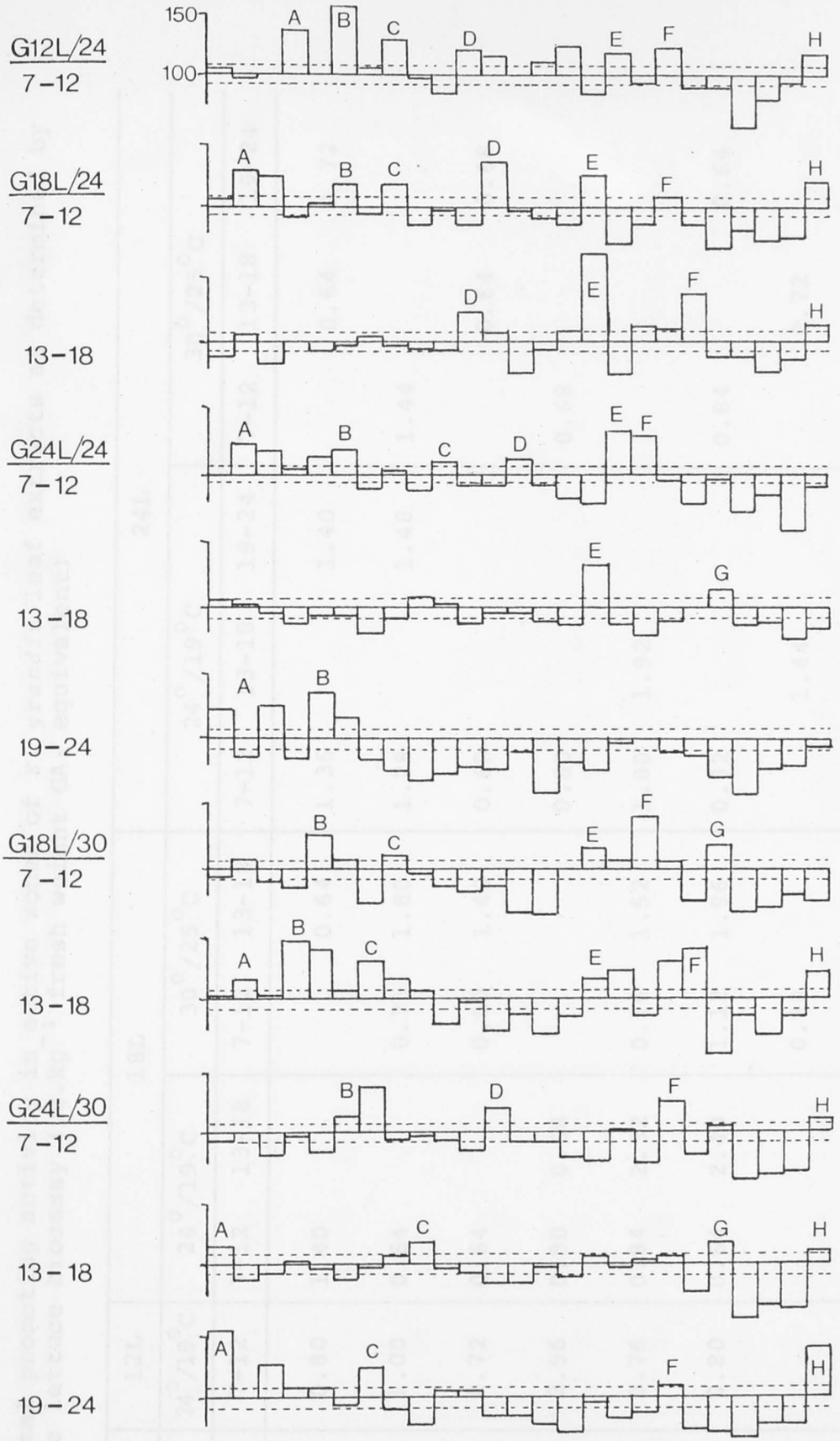


Figure 9-1. (continued)

Table 9-1. Total promoting activity in active zones of *E. grandis* leaf extracts as determined by the lettuce bioassay ($\mu g.kg^{-1}$ fresh weight GA_3 equivalent)

HARVEST	12L	18L				24L					
TEMPERATURE	24°/19°C	24°/19°C		30°/25°C		24°/19°C			30°/25°C		
LEAVES	7-12	7-12	13-18	7-12	13-18	7-12	13-18	19-24	7-12	13-18	19-24
ZONE A FRACTIONS 1-4	0.80	1.40			0.64	1.36		1.40		0.64	1.72
ZONE B FRACTIONS 5-7	1.00	0.64		0.76	1.80	1.28		1.48	1.44		
ZONE C FRACTIONS 8-10	0.72	0.64		0.60	1.40	0.60				0.64	0.68
ZONE D FRACTIONS 11-15	2.56	0.80	0.68			0.60			0.68		
ZONE E FRACTIONS 16-17	0.76	0.84	2.32	0.76	1.52	1.00	1.92				
ZONE F FRACTIONS 18-20	0.80	0.64	2.40	1.12	1.96	0.92			0.84		0.64
ZONE G FRACTIONS 21-23				0.80			1.44			0.72	
ZONE H FRACTIONS 24-25	0.76	0.80	0.68		0.76				0.68	0.68	1.04
TOTAL	7.40	5.76	6.08	4.04	8.08	5.76	3.36	2.88	3.64	2.68	4.08

Table 9-2. Total promoting activity in active zones of *E. regnans* leaf extracts as determined by the lettuce bioassay ($\mu g.kg^{-1}$ fresh weight GA_3 equivalent)

HARVEST	12L	18L				24L					
TEMPERATURE	24°/19°C	24°/19°C		30°/25°C		24°/19°C			30°/25°C		
LEAVES	7-12	7-12	13-18	7-12	13-18	7-12	13-18	19-24	7-12	13-18	19-24
ZONE A FRACTIONS 1-4	1.77	1.64	2.08	1.28	2.00	2.28	1.68	1.16	0.76	1.28	0.72
ZONE B FRACTIONS 5-7	0.78	1.20				1.20		1.44	0.60	1.12	0.96
ZONE C FRACTIONS 8-10		0.68		0.80	0.68	0.84	1.20	1.36	1.44		
ZONE D FRACTIONS 11-15	2.55	1.28	2.16			0.60	1.48	0.76	1.24		1.48
ZONE E FRACTIONS 16-17	2.08	2.16				3.56		1.68		1.00	
ZONE F FRACTIONS 18-20	1.25		0.72	0.92	1.92	0.68	0.72	0.68		0.80	0.68
ZONE G FRACTIONS 21-23		1.92	1.64			0.80	0.72				0.68
ZONE H FRACTIONS 24-25			0.72			0.72	0.72				
TOTAL	8.43	8.88	7.32	3.00	4.60	10.68	6.52	7.08	4.04	4.20	4.52

Table 9-3. Total inhibiting activity in active zones of *E. grandis* leaf extracts as determined by the cress bioassay. ($\mu g.kg^{-1}$ fresh weight ABA equivalents)

HARVEST	12L	18L				24L					
TEMPERATURE	24°/19°C	24°/19°C		30°/25°C		24°/19°C			30°/25°C		
LEAVES	7-12	7-12	13-18	7-12	13-18	7-12	13-18	19-24	7-12	13-18	19-24
ZONE A FRACTIONS 1-3		34.4			19.6				19.6	14.8	
ZONE B FRACTIONS 4-6				14.8	19.6				50.8	71.2	26.4
ZONE C FRACTIONS 7-9		51.6	14.8	29.6	14.8	36.0				39.2	
ZONE D FRACTIONS 10-11		26.4	14.8		14.8	14.8					183.6
ZONE E FRACTIONS 12-15	14.8	49.2	14.8	52.8	14.8			55.6	14.8	19.6	>350.8
ZONE F FRACTIONS 20-24	90.8	>350.8	>480.0	66.4	>494.8	>274.4	103.2	>531.6	>514.4	>302.4	>358.8
TOTAL ZONES A-E	14.8	161.6	44.4	97.2	83.6	50.8	-	55.6	85.2	144.8	>560.8
TOTAL ZONES A-F	105.6	>512.4	>524.4	163.6	>578.4	>325.2	103.2	>587.2	>599.6	>447.2	>919.6

Table 9-4. Total inhibiting activity in active zones of *E. regnans* leaf extracts as determined by the cress bioassay ($\mu g.kg^{-1}$ fresh weight ABA equivalent)

HARVEST	12L	18L				24L					
TEMPERATURE	24°/19°C	24°/19°C		30°/25°C		24°/19°C			30°/25°C		
LEAVES	7-12	7-12	13-18	7-12	13-18	7-12	13-18	19-24	7-12	13-18	19-24
ZONE A FRACTIONS 1-3	34.4		26.4	14.8	19.6	52.8		14.8		14.8	
ZONE B FRACTIONS 4-6	25.5	14.8	41.2	14.8	14.8	19.6					
ZONE C FRACTIONS 7-9	25.5	36.0				41.2			34.4		
ZONE D FRACTIONS 10-11	19.3	19.6		29.6		26.4		50.8			14.8
ZONE E FRACTIONS 12-15	44.8		14.8	26.4	29.6	66.4				14.8	
ZONE F FRACTIONS 20-24	34.4						>480	125.6	>289.2	>259.6	72.0
TOTAL ZONES A-E	149.5	70.4	82.4	85.6	64.0	206.4	-	65.6	34.4	29.6	14.8
TOTAL ZONES A-F	183.9	70.4	82.4	85.6	64.0	206.4	>480	191.2	>323.6	>289.2	86.8

a particular stage of development can be seen from a comparison of the 7-12 leaves harvested at the 12, 18 and 24 leaf-pair stages, and of the 13-18 leaves at the 18 and 24 leaf-pair stages, within each species-temperature group. To facilitate such comparisons the total GA_3 equivalent concentration for each set of leaves is recorded in Table 9-5: in this Table, leaf age effects are responsible for the differences across horizontal rows in each species-temperature group.

Table 9-5. Total activity of gibberellin-like promoters for each bioassay, in µg/kg fresh weight GA_3 equivalent.

	24°/19°C			30°/25°C		
LEAVES	12L	18L	24L	12L	18L	24L
E. grandis						
7-12	7.4	5.8	5.8	-	4.0	3.6
13-18		6.1	3.4		8.1	2.7
19-24			2.9			4.1
E. regnans						
7-12	8.4	8.9	10.7	-	3.0	4.0
13-18		7.3	6.5		4.6	4.2
19-24			7.1			4.5

The effect of leaf aging on total promoter concentration appears to be independent of temperature, the same trends occurring at both 24°/19°C and 30°/25°C for each species. In *E. grandis*, concentrations fall with increasing age in both

the 7-12 and 13-18 leaves. In *E. regnans* increasing age has only a small effect in the 13-18 leaves, but a distinct increase in concentration occurs with aging in the 7-12 leaves; such a build-up in only the older leaves of the plant seems likely to result from the storage there of an unwanted metabolic product. The two species thus differ in the effect of aging on the quantity of promoters in their leaves, and this effect also appears to depend on the position of the leaves in the tree.

Table 9-6. Concentration of promoters in active zones of *E. grandis*: comparison of leaf age effects. ($\mu g.kg^{-1}$ fresh weight GA_3 equivalent)

	Active Zone							
	A	B	C	D	E	F	G	H
7-12 leaves								
12L/24	0.80	1.00	0.72	2.56	0.76	0.80		0.76
18L/24	1.40	0.64	0.64	0.80	0.84	0.64		0.80
24L/24	1.36	1.28	0.60	0.60	1.00	0.92		
18L/30		0.76	0.60		0.76	1.12	0.80	
24L/30		1.44		0.68		0.84		0.68
13-18 leaves								
18L/24				0.68	2.32	2.40		0.68
24L/24					1.92		1.44	
18L/30	0.64	1.80	1.40		1.52	1.96		0.76
24L/30	0.64		0.64				0.72	0.68

These effects on total concentration of gibberellin-like promoters can be analysed in more detail by reference to the data for individual active zones in Tables 9-6 and 9-7. Interpretation of the data is made difficult by the effects of the complex interactions discussed in the previous section, but

a number of features are worthy of comment. The sharp decrease in total promoter concentration with increasing leaf age in the 13-18 leaves of *E. grandis* is attributable largely to the removal or breakdown of two promoters, zones E and F, with an additional contribution from zone B at $30^{\circ}/25^{\circ}C$. In the 7-12 leaves the decrease in total promoter concentration with leaf age was less marked, and the trends in individual zones are variable. The relatively high concentration in leaves from the 12L harvest at $24^{\circ}/19^{\circ}C$ can be seen to result mainly from a compound in zone D. Promotion in zones E and F is weaker than in the 13-18 leaves and does not show the decline with leaf age observed there.

Table 9-7. Concentration of promoters in active zones of *E. regnans*: comparison of leaf age effects. ($\mu g.kg^{-1}$ fresh weight GA_3 equivalent)

	Active Zone							
	A	B	C	D	E	F	G	H
7-12 leaves								
12L/24	1.77	0.78		2.55	2.08	1.25		
18L/24	1.64	1.20	0.68	1.28	2.16		1.92	
24L/24	2.28	1.20	0.84	0.60	3.56	0.68	0.80	0.72
18L/30	1.28		0.80			0.92		
24L/30	0.76	0.60	1.44	1.24				
13-18 leaves								
18L/24	2.08			2.16		0.72	1.64	0.72
24L/24	1.68		1.20	1.48		0.72	0.72	0.72
18L/30	2.00		0.68			1.92		
24L/30	1.28	1.12			1.00	0.80		

The declining concentration in the 13-18 leaves of *E. regnans* is caused in part by a reduction in the activity of zone A, with contributions from zones D and G at $24^{o}/19^{o}C$ and zones C and F at $30^{o}/25^{o}C$. Thus, although the trend in total promoter concentration in these leaves is the same in both species the compounds involved are different. The increasing total concentration in the 7-12 leaves results from increases in all zones except D and F at $24^{o}/19^{o}C$, zones A and E being the major contributors; at $30^{o}/25^{o}C$ zone E is inactive and the overall increase in activity is largely due to zone D, with minor contributions from B and C.

In spite of the anomalies present in the data for individual zones, the foregoing analysis enables some qualification of the conclusions derived from the total concentration data. In the first place, the effect of leaf age on the concentrations of the promoting compounds present is the same at both temperatures in only a few cases, e.g. zone F in *E. regnans* and zone C in *E. grandis*. Secondly, the two species differ in the qualitative as well as quantitative effects of aging on promoter concentrations; there are also some species similarities, notably in zone D. On the whole, the effects of age on total promoter concentrations are attributable mainly to effects on zones A, B, D, E, and F.

9.2.1.2. Effects of seedling age

By following the trend in concentration of promoting substances from the 7-12 leaves at the 12 leaf-pair harvest, through the 13-18 leaves at the 18 leaf-pair harvest to the 19-24 leaves at the 24 leaf-pair harvest, i.e. the youngest leaves

at each stage, an indication of the effects of seedling age on the presence of these substances can be gained. Similarly, the 7-12 leaves at the 18 leaf-pair stage can be compared with the 13-18 leaves at the 24 leaf-pair stage. Any changes noted in this way reflect a change in the physiology of the seedlings with increasing maturity, and whereas the changes in concentration with increasing leaf age described in the previous section were due to translocation or metabolism of existing reserves, this section is concerned with differences in the amount of promoting substances originally present in the newly initiated leaves.

A study of the total promoter concentrations in Table 9-5 (reading diagonally within each species-temperature group) reveals a remarkably uniform decrease with increasing seedling age in all four groups. The decline in concentration is steeper in *E. grandis* than in *E. regnans*, especially at $30^{o}/25^{o}C$ where the one exception occurs - an increase in concentration from the 7-12 leaves at the 18 leaf-pair stage to the 13-18 leaves at the 24 leaf-pair stage. The lack of replication of the bioassays makes the reality of this apparent exception uncertain, but it is clear that a difference between the two species exists in the extent to which concentrations decline up to the 24 leaf-pair stage.

The analysis of these effects in terms of individual active zones (Tables 9-8 and 9-9) is again confused somewhat by the nature of the data, but may nonetheless provide further information on the effects of age on promoter concentrations and their interactions with the effects of growing temperature. The decline in total promoter concentration in

E.grandis at 24°/19°C is due largely to the disappearance of zone D, with other contributions from zones C, F, and H. The absence of activity in zone E in the 19-24 leaves from the 24 leaf-pair harvest at this temperature is suspect - the other data for this zone lead one to expect considerable activity there, but the bioassay results in Figure 9-1 reveal that inhibition rather than promotion occurred in fractions 16-17; as this is the only instance of inhibition recorded in these fractions for either species the results for zone E in this particular bioassay are doubtful. At 30°/25°C, zone D is absent from *E. grandis* but zones C and F again decrease in concentration with increasing age; zones B and E also decline, in contrast to their apparent behaviour at 24°/19°C. The reverse occurs in zone H, which increases in concentration with age at 30°/25°C rather than decreasing as at 24°/19°C.

Table 9-8. Concentration of promoters in active zones of *E. grandis*: comparison of seedling age effects. ($\mu g.kg^{-1}$ fresh weight GA_3 equivalent)

	Active Zone							
	A	B	C	D	E	F	G	H
youngest leaves								
12L/24: 7-12	0.80	1.00	0.72	2.56	0.76	0.80		0.76
18L/24:13-18				0.68	2.32	2.40		0.68
24L/24:19-24	1.40	1.48						
18L/30:13-18	0.64	1.80	1.40		1.52	1.96		0.76
24L/30:19-24	1.72		0.68			0.64		1.04
older leaves								
18L/24: 7-12	1.40	0.64	0.64	0.80	0.84	0.64		0.80
24L/24:13-18					1.92		1.44	
18L/30: 7-12		0.76	0.60		0.76	1.12	0.80	
24L/30:13-18	0.64		0.64				0.72	0.68

Table 9-9. Concentration of promoters in active zones of *E. regnans*: comparison of seedling age effects. ($\mu g.kg^{-1}$ fresh weight GA_3 equivalent)

	Active Zone							
	A	B	C	D	E	F	G	H
youngest leaves								
12L/24: 7-12	1.77	0.78		2.55	2.08	1.25		
18L/24:13-18	2.08			2.16		0.72	1.64	0.72
24L/24:19-24	1.16	1.44	1.36	0.76	1.68	0.68		
18L/30:13-18	2.00		0.68			1.92		
24L/30:19-24	0.72	0.96		1.48		0.68	0.68	
older leaves								
18L/24: 7-12	1.64	1.20	0.68	1.28	2.16		1.92	
24L/24:13-18	1.68		1.20	1.48		0.72	0.72	0.72
18L/30: 7-12	1.28		0.80			0.92		
24L/30:13-18	1.28	1.12			1.00	0.80		

In the upper leaves of *E. regnans* at 24°/19°C zones D, F, and H again are partly responsible for the decrease in total concentration, but other zones also contribute. The effects of all these are reduced somewhat by strong increases in zones B and C, leading to the less marked decline in total concentration with seedling age in this species compared with *E. grandis*. At 30°/25°C zones A, C and F decrease, but strong increases in zones B, D and E bring about the increase in total concentration mentioned previously. Once again the analysis of changes in the concentration of promoters in individual zones indicates that the overall trends are by no means common to all the active compounds present, and that real differences between the two temperature regimes exist in both species.

[1] As indicated by the diagram facing page 167, the terms "up the tree" and "down the tree" used here and in subsequent description of positional effects do not adequately reflect the differing positions of leaf samples being compared. In fact, the strata of leaves sampled within each seedling were a series of overlapping cones, so strictly speaking the variations described as "up the tree" are variations from the inner to the outer cone.

9.2.1.3. Effects of position in the seedling

The two types of age effects discussed above, namely changes in the original concentration of active substances in the leaves as the plant matures and changes in the concentration of those substances in a particular leaf as it ages, act together to determine the way in which concentrations vary with the position of a leaf in a seedling at any given time. In addition at least one case of interaction of the two age effects is evident in the data of Table 9-5, as discussed in Section 9.2.1.1. In seedling material such as that used here, it is unlikely that the physical position of a leaf has any effect on its content of growth substances, although in larger plants it is possible that such effects could arise through shading of the lower or inner leaves by higher or outer ones. The differences in distribution of promoters and inhibitors observed here in seedlings of a given age are thus the combination of age effects on the seedling and its leaves.

The variation in total concentration of promoters with position in the tree is the same for both species, but is dependent on the temperature regime (Table 9-5, reading vertically within each species-temperature group). At $24^{\circ}/19^{\circ}C$, concentrations are highest in the lower leaves, decreasing up the tree.[1] At $30^{\circ}/25^{\circ}C$ this effect is reversed so that concentrations decrease down the tree, although the amount of variation with position in the tree is generally small at this temperature.

Table 9-10. Concentration of promoters in active zones of *E. grandis*: comparison of leaf position effects. ($\mu g.kg^{-1}$ fresh weight GA_3 equivalent)

	Active Zones							
	A	B	C	D	E	F	G	H
24L harvest								
24L/24: 7-12	1.36	1.28	0.60	0.60	1.00	0.92		
24L/24:13-18					1.92		1.44	
24L/24:19-24	1.40	1.48						
24L/30: 7-12		1.44		0.68		0.84		0.68
24L/30:13-18	0.64		0.64				0.72	0.68
24L/30:19-24	1.72		0.68			0.64		1.04
18L harvest								
18L/24: 7-12	1.40	0.64	0.64	0.80	0.84	0.64		0.80
18L/24:13-18				0.68	2.32	2.40		0.68
18L/30: 7-12		0.76	0.60		0.76	1.12	0.80	
18L/30:13-18	0.64	1.80	1.40		1.52	1.96		0.76

Table 9-11. Concentration of promoters in active zones of *E. regnans*: comparison of leaf position effects. ($\mu g.kg^{-1}$ fresh weight GA_3 equivalent)

	Active Zones							
	A	B	C	D	E	F	G	H
24L harvest								
24L/24: 7-12	2.28	1.20	0.84	0.60	3.56	0.68	0.80	0.72
24L/24:13-18	1.68		1.20	1.48		0.72	0.72	0.72
24L/24:19-24	1.16	1.44	1.36	0.76	1.68	0.68		
24L/30: 7-12	0.76	0.60	1.44	1.24				
24L/30:13-18	1.28	1.12			1.00	0.80		
24L/30:19-24	0.72	0.96		1.48		0.68	0.68	
18L harvest								
18L/24: 7-12	1.64	1.20	0.68	1.28	2.16		1.92	
18L/24:13-18	2.08			2.16		0.72	1.64	0.72
18L/30: 7-12	1.28		0.80			0.92		
18L/30:13-18	2.00		0.68			1.92		

As usual, a consideration of the concentrations of individual active zones (Tables 9-10 and 9-11) reveals that there are important differences between the species which do not appear in the total concentration effects. In *E. regnans* at the 24 leaf-pair stage, the decrease in promoter concentration up the tree at $24^{o}/19^{o}$C is due to decreases in zones A, E and G, but in *E. grandis* the overall decrease is attributable to zones C, D, E and F. At $30^{o}/25^{o}$C, zone E is weak or inactive at the 24 leaf-pair stage and in spite of the repeated small decline in concentration of some zones, the increases in zones B, D, F and G of *E. regnans* and zones A, C and H of *E. grandis* lead to an increase in total concentration.

9.2.1.4. Effects of species and temperature

The most direct way to examine the effects of temperature on promoter concentrations in each species in isolation from the age effects is to sum the concentrations of each zone over the five observations within each species-temperature group, omitting the 12 leaf-pair harvest data for $24^{o}/19^{o}$C in order to enable unbiassed comparisons with the $30^{o}/25^{o}$C data. The totals obtained in this way for each active zone are presented in Table 9-12. Although the figures in this Table, as sums of the biological activities of different leaf extracts, have no real meaning, they do provide a basis for comparisons between the two species and two temperature regimes used.

Table 9-12. Pooled concentration of promoters in each active zone over five bioassays. ($\mu g.kg^{-1}$ F.W. GA_3 equivalent)

ACTIVE ZONE	*E. grandis*		*E. regnans*	
	24°/19°C	30°/25°C	24°/19°C	30°/25°C
A	4.2	3.0	8.8	6.0
B	3.4	4.0	3.8	2.7
C	1.2	3.3	4.1	2.9
D	2.1	0.7	6.3	2.7
E	6.1	2.3	7.4	1.0
F	4.0	4.6	2.8	4.3
G	1.4	1.5	5.1	0.7
H	1.5	3.2	2.2	-
TOTAL	23.9	22.6	40.5	20.3

Considering first the total promoter concentrations in each group, it is obvious that *E. regnans* contains rather higher quantities of promoting substances at 24°/19°C, where both species grow well. An increase in temperature of 6°C reduces the concentration in *E. regnans* by about 50%, while having little effect on total concentration in *E. grandis*. This can be compared with the effects of temperature on the growth of these species, described in Chapter 3: while *E. grandis* remains vigorous (in fact becomes more so) as day temperature is increased from 24°C to 30°C, the growth of *E. regnans* declines markedly. Unfortunately the lack of data on promoter concentrations in very young seedlings makes it impossible to say whether promoter concentrations in

E. regnans at 30°/25°C are initially as high as at 24°/19°C, during the period when growth is equally vigorous under both temperature regimes.

Comparisons of the pooled data for individual active zones between the two species indicate that most of the extra activity in *E. regnans* at 24°/19°C is located in zones A and D, with smaller contributions from zones C and G. Levels of B, E and H are about the same in both species, while *E. grandis* has more activity in zone F.

The similarities and differences between the species in the effects of temperature on individual active zones can be clearly discerned. The concentration of promoters in zones A, D and E falls with increasing temperature in both species; in fact, allowing for some discrepancy in the allocation of bioassay responses to active zones, the data of Tables 9-1 and 9-2 suggest that zone D in *E. grandis* and zone E in *E. regnans* may be inactive at 30°/25°C. Conversely, zone F shows a distinct increase in concentration with increasing temperature in both species. Zones B, C, G and H differ between the two species, B and C tending to rise in *E. grandis* and fall in *E. regnans* with increasing temperature while the concentration of zone G,never important in *E. grandis*, remains the same there and that of zone H increases; in *E. regnans* H and probably G also are inactive at 30°/25°C. If the difference in promoter concentrations in this species at the two temperatures examined is responsible in part for the observed difference in health and vigour of the seedlings, then it is likely that the disappearance of the active substances from zones G and H is important in causing this effect.

9.2.1.5. Summary of results on growth promoters

(i) As individual leaves age in *E. grandis* the gibberellin content declines; in *E. regnans* the gibberellin content increases with increasing leaf age.

(ii) The gibberellin content of newly initiated leaves in *E. grandis* decreases with increasing seedling age; the decline is less marked in *E. regnans*, particularly when the seedlings are grown at $30^{\circ}/25^{\circ}C$.

(iii) The gibberellin content of leaves of *E.grandis* and *E. regnans* declines with position up the stem in seedlings grown at $24^{\circ}/19^{\circ}C$; at $30^{\circ}/25^{\circ}C$ the leaf gibberellin content increases with position up the stem.[1]

(iv) *E. regnans* leaf extracts contain considerably more gibberellin-like promoting activity than *E. grandis* extracts when grown at $24^{\circ}/19^{\circ}C$ but when grown at the higher temperature of $30^{\circ}/25^{\circ}C$ the total promoting activity in *E. regnans* is reduced by about 50%; the activity in *E. grandis* is unchanged.

(v) Differences in gibberellin-like substances between the species, between leaf types and between temperature regimes are complex and could involve a variety of different gibberellins.

9.2.2. Growth inhibiting substances

The effects of age, temperature and species on the concentration of inhibitors in the leaves can be examined in the same way as the effects on promoter concentrations.

[1]See footnote to p. 195.

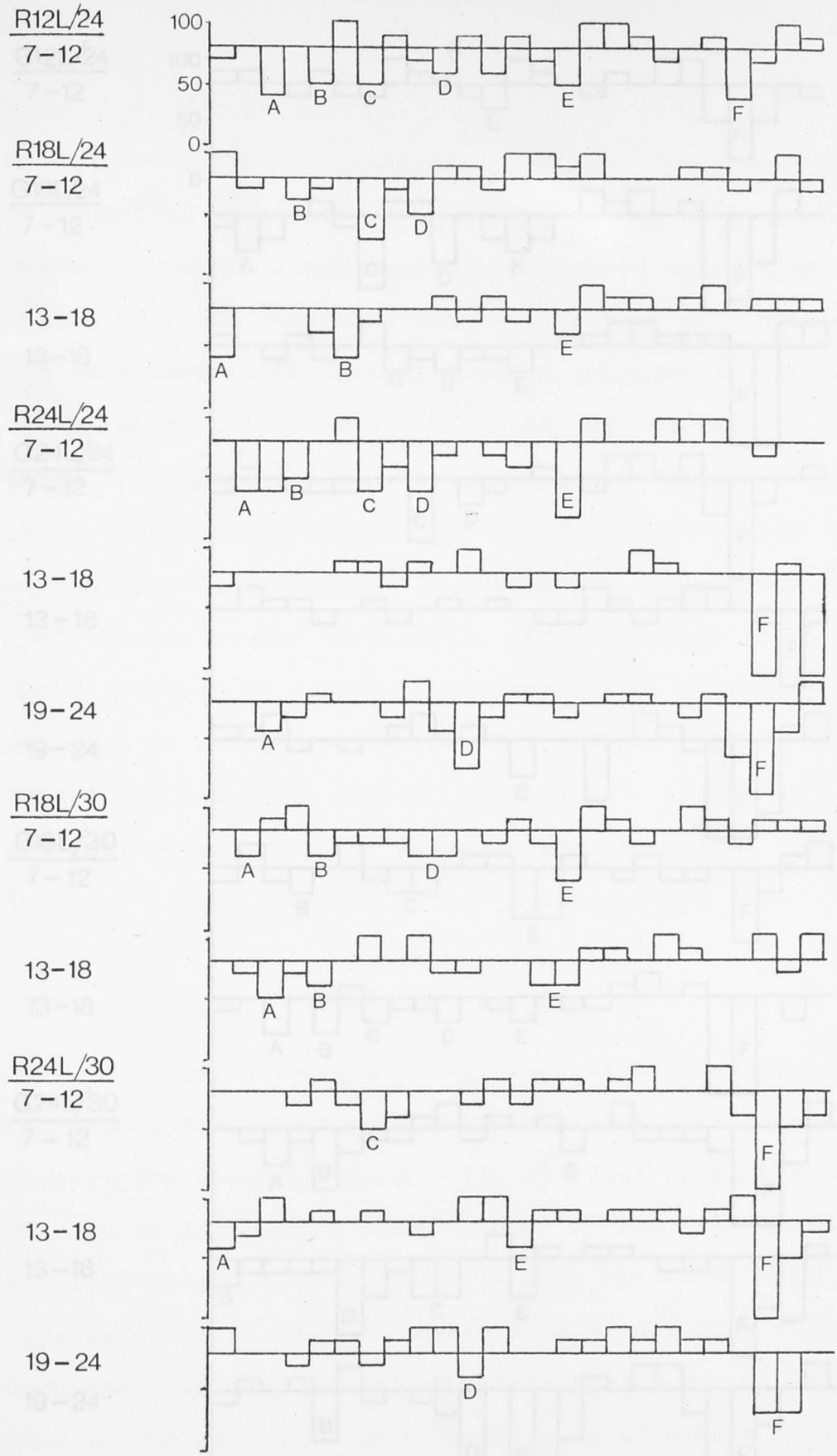


Figure 9-2. Histogrammed results of cress bioassays of 22 leaf extracts. Ordinate: percentage of control germination. Abscissa: fractions 1-25.

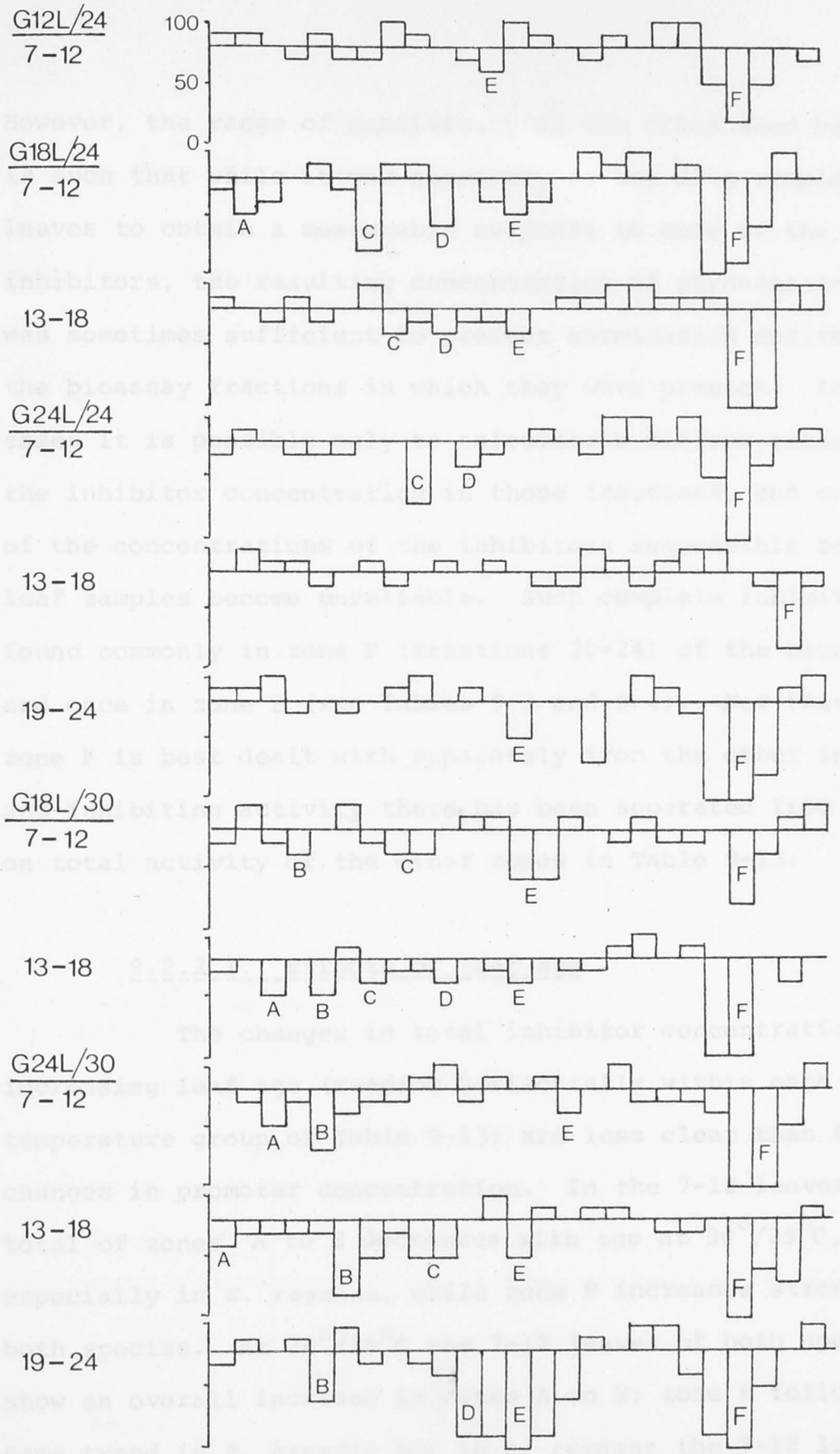


Figure 9-2. (continued)

However, the range of sensitivity of the cress seed bioassay is such that while it was necessary to use 25 g samples of leaves to obtain a measurable response to some of the weaker inhibitors, the resulting concentration of stronger inhibitors was sometimes sufficient to prevent germination entirely in the bioassay fractions in which they were present. In such cases it is possible only to calculate a minimum estimate of the inhibitor concentration in those fractions, and comparisons of the concentrations of the inhibitors responsible between leaf samples become unreliable. Such complete inhibition was found commonly in zone F (fractions 20-24) of the bioassays and once in zone E (see Tables 9-3 and 9-4). For this reason zone F is best dealt with separately from the other inhibitors, and inhibiting activity there has been separated from data on total activity of the other zones in Table 9-13.

9.2.2.1. Effects of leaf age

The changes in total inhibitor concentration with increasing leaf age (reading horizontally within each species-temperature group of Table 9-13) are less clear than the changes in promoter concentration. In the 7-12 leaves, the total of zones A to E decreases with age at $30^{o}/25^{o}C$, especially in *E. regnans*, while zone F increases strongly in both species. At $24^{o}/19^{o}C$ the 7-12 leaves of both species show an overall increase in zones A to E; zone F follows the same trend in *E. grandis* but in *E. regnans* the 7-12 leaves only show slight activity in this zone at the 12 leaf-pair stage, disappearing by the next harvest. Inhibitor concentrations in the 13-18 leaves do not follow the same trends

with age as in the 7-12 leaves; note particularly the increase in zone F in *E. regnans* at 24°/19°C.

Table 9-13. Total activity of acid inhibitors for each bioassay, in $\mu g.kg^{-1}$ F.W. ABA equivalent

LEAVES	24°/19°C			30°/25°C		
	12L	18L	24L	12L	18L	24L
(i) Zones A to E						
E. grandis						
7-12	14.8	161.6	50.8	-	97.2	85.2
13-18		44.4	0.0		83.6	144.8
19-24			55.6			>560.8
E. regnans						
7-12	149.5	70.4	206.4	-	85.6	34.4
13-18		82.4	0.0		64.0	29.6
19-24			65.6			14.8
(ii) Zone F						
E. grandis						
7-12	90.8	>350.8	>274.4	-	66.4	>514.4
13-18		>480.0	103.2		>494.8	>302.4
19-24			>531.6			>358.8
E. regnans						
7-12	34.4	-	-	-	-	>289.2
13-18		-	>480.0		-	>259.6
19-24			125.6			72.0

An inspection of the data for individual zones in Tables 9-14 and 9-15 adds little to the age trends suggested by the total data of Table 9-13, except to indicate once again that the overall trends do not apply to every active compound present, and that species differences in the effects of temperature on individual zones exist even where the same trend in total concentration occurs.

Table 9-14. Concentration of inhibitors in active zones of *E. grandis*: comparison of leaf age effects. ($\mu g.kg^{-1}$ fresh weight ABA equivalent)

	Active Zone					
	A	B	C	D	E	F
7-12 leaves						
12L/24					14.8	90.8
18L/24	34.4		51.6	26.4	49.2	>350.8
24L/24			36.0	14.8		>274.4
18L/30		14.8	29.6		52.8	66.4
24L/30	19.6	50.8			14.8	>514.4
13-18 leaves						
18L/24			14.8	14.8	14.8	>480.0
24L/24						103.2
18L/30	19.6	19.6	14.8	14.8	14.8	>494.8
24L/30	14.8	71.2	39.2		19.6	>302.4

Table 9-15. Concentration of inhibitors in active zones of *E. regnans*: comparison of leaf age effects. ($\mu g.kg^{-1}$ fresh weight ABA equivalent)

	Active Zone					
	A	B	C	D	E	F
7-12 leaves						
12L/24	34.4	25.5	25.5	19.3	44.8	34.4
18L/24		14.8	36.0	19.6		
24L/24	52.8	19.6	41.2	26.4	66.4	
18L/30	14.8	14.8		29.6	26.4	
24L/30			34.4			>289.2
13-18 leaves						
18L/24	26.4	41.2			14.8	
24L/24						>480.0
18L/30	19.6	14.8			29.6	
24L/30	14.8				14.8	>259.6

9.2.2.2. Effects of seedling age

The effects of increasing maturity of the seedlings, seen from a comparison of concentrations in the upper leaves at each harvest, (reading diagonally within each species-temperature group of Table 9-13) are more distinct than the effects of leaf age on the quantities of inhibitors present. In *E. grandis* the total inhibitor concentration excepting zone F increases with increasing seedling age at both temperatures, but much more so at $30^{o}/25^{o}$C. In *E.regnans* the inhibitor concentration decreases steadily with age at both temperatures. The concentration of zone F increases with seedling age in both species and both temperature regimes.

Table 9-16. Concentration of inhibitors in active zones of *E. grandis*: comparison of seedling age effects. ($\mu g.kg^{-1}$ fresh weight ABA equivalent)

	Active Zone					
	A	B	C	D	E	F
youngest leaves						
12L/24: 7-12					14.8	90.8
18L/24:13-18			14.8	14.8	14.8	>480.0
24L/24:19-24					55.6	>531.6
18L/30:13-18	19.6	19.6	14.8	14.8	14.8	>494.8
24L/30:19-24		26.4		183.6	>350.8	>358.8
older leaves						
18L/24: 7-12	34.4		51.6	26.4	49.2	>350.8
24L/24:13-18						103.2
18L/30: 7-12		14.8	29.6		52.8	66.4
24L/30:13-18	14.8	71.2	39.2		19.6	>302.4

Table 9-17. Concentration of inhibitors in active zones of *E. regnans*: comparison of seedling age effects. ($\mu g.kg^{-1}$ fresh weight ABA equivalent)

	Active Zone					
	A	B	C	D	E	F
youngest leaves						
12L/24: 7-12	34.4	25.5	25.5	19.3	44.8	34.4
18L/24:13-18	26.4	41.2			14.8	
24L/24:19-24	14.8			50.8		125.6
18L/30:13-18	19.6	14.8			29.6	
24L/30:19-24				14.8		72.0
older leaves						
18L/24: 7-12		14.8	36.0	19.6		
24L/24:13-18						>480.0
18L/30: 7-12	14.8	14.8		29.6	26.4	
24L/30:13-18	14.8				14.8	>259.6

[1]See footnote to p. 195.

Analysis of the effects on zones A to E in terms of the data of Tables 9-16 and 9-17 reveals that the increase in total activity with age in the youngest leaves of *E. grandis* at 24°/19°C is due to an increase in zone E. At 30°/25°C this increase is stronger, and is accompanied by a large increase in concentration in zone D. In *E. regnans* all of zones A to E decrease in activity with increasing seedling age except zone D in the upper leaves, which shows small increases under both temperature regimes, hidden in the total data by the decline of inhibition in all other zones.

9.2.2.3. Effects of position in the seedling

The combined effects of age of the leaves and whole seedlings are reflected in the variation in inhibitor concentrations with position in the seedling, as discussed in Section 9.2.1.3. These effects can be seen from vertical comparisons within each species-temperature group of Table 9-13. At the 24 leaf-pair stage, total inhibitor concentration in zones A to E decreases up the seedling[1] in *E. regnans* at both temperatures, while in *E. grandis* the opposite trend is very strong at 30°/25°C, and little overall variation with position in the tree is seen at 24°/19°C. The absence of significant inhibition from both species in the 13-18 leaves at the 24 leaf-pair harvest may be caused by the translocation of inhibitors from maturing leaves to older parts of the plant, where they may be implicated in the processes of senescence and abscission. Zone F shows much the same trends up the tree as zones A to E.

At the 18 leaf-pair harvest inhibitor concentrations in zones A to E decreased up the tree in *E. grandis* at both temperatures and in *E. regnans* at 30°/25°C, but not at 24°/19°C in contrast to the decrease at the 24 leaf-pair stage. The concentration of inhibitors in zone F was stable except in *E. grandis* at 30°/25°C where it increased considerably up the tree. The major differences in distribution of inhibitors between species at both harvests can be attributed to the difference in effect of seedling age described above, whereby concentrations rise in *E. grandis* and fall in *E. regnans*.

Table 9-18. Concentration of inhibitors in active zones of *E. grandis*: comparison of leaf position effects. ($\mu g.kg^{-1}$ fresh weight ABA equivalent)

	Active Zone					
	A	B	C	D	E	F
24L harvest						
24L/24: 7-12			36.0	14.8		>274.4
24L/24:13-18						103.2
24L/24:19-24					55.6	>531.6
24L/30: 7-12	19.6	50.8			14.8	>514.4
24L/30:13-18	14.8	71.2	39.2		19.6	>302.4
24L/30:19-24		26.4		183.6	>350.8	>358.8
18L/24: 7-12	34.4		51.6	26.4	49.2	>350.8
18L/24:13-18			14.8	14.8	14.8	>480.0
18L/30: 7-12		14.8	29.6	52.8	52.8	66.4
18L/30:13-18	19.6	19.6	14.8	14.8	14.8	>494.8

Table 9-19. Concentration of inhibitors in active zones of *E. regnans*: comparison of leaf position effects. ($\mu g.kg^{-1}$ fresh weight ABA equivalent)

	Active Zone					
	A	B	C	D	E	F
24L harvest						
24L/24: 7-12	52.8	19.6	41.2	26.4	66.4	
24L/24:13-18						>480.0
24L/24:19-24	14.8			50.8		125.6
24L/30: 7-12			34.4			>289.2
24L/30:13-18	14.8				14.8	>259.6
24L/30:19-24				14.8		72.0
18L harvest						
18L/24: 7-12		14.8	36.0	19.6		
18L/24:13-18	26.4	41.2			14.8	
18L/30: 7-12	14.8	14.8		29.6	26.4	
18L/30:13-18	19.6	14.8			29.6	

The data for individual zones (Tables 9-18 and 9-19) reveal that zone E is again largely responsible for the trends seen in total inhibitor content. At the 24 leaf-pair stage concentrations in this zone increase up the tree in *E. grandis*, especially at $30^{\circ}/25^{\circ}C$, but in *E. regnans* they decrease up the tree. Again only zone D in *E. regnans* increases up the tree at this harvest, in both temperature regimes. Conversely at the 18 leaf-pair stage zone E decreases up the tree in *E. grandis* and increases in *E. regnans*; the increase is more than balanced by a decrease in zone D at $30^{\circ}/25^{\circ}C$, but at $24^{\circ}/19^{\circ}C$ additional increases in zones A and B are not offset by the reduction in activity of zones C and D, and a net increase in total inhibition occurs as described above.

9.2.2.4. Effects of species and temperature

To facilitate comparisons between species and temperature regimes the inhibitor concentration data from each bioassay have been pooled in the same way as the promoter data were, and the totals for active zones A to F are shown in Table 9-20. It is immediately apparent from the Table that there is a profound difference between species in their response to a rise in temperature from $24^{\circ}/19^{\circ}C$ to $30^{\circ}/25^{\circ}C$. The total inhibitor concentration in zones A to E of *E. regnans* falls by almost 50%, but in *E. grandis* the concentration is more than trebled. It is important to note that this temperature effect is dependent on tree age: a similar pooling of data at the 18 leaf-pair stage shows little difference between temperature regimes for either species.

As has already become obvious from the discussion of seedling age effects, zone E is primarily responsible for the much higher content of inhibitors in *E. grandis* at $30^{\circ}/25^{\circ}C$ than at $24^{\circ}/19^{\circ}C$. Large differences in concentration in zones B and D also make an important contribution to the effect, while only zone C shows a lower activity at the higher temperature. In *E. regnans* all of zones A to E show a decrease in activity at $30^{\circ}/25^{\circ}C$ compared to $24^{\circ}/19^{\circ}C$. In zone F, there appears to be little difference in concentration between temperature regimes in either species, but there is a large difference between species, with *E. grandis* having possibly as much as three times the activity of *E. regnans*.

Table 9-20. Pooled concentration of inhibitors in each active zone over five bioassays. ($\mu g.kg^{-1}$ F.W. ABA equivalent)

ACTIVE ZONE	*E. grandis*		*E. regnans*	
	24°/19°C	30°/25°C	24°/19°C	30°/25°C
A	34.4	54.0	94.0	49.2
B	-	182.8	75.6	29.6
C	102.4	83.6	77.2	34.4
D	56.0	198.4	96.8	44.4
E	119.6	>452.8	81.2	70.8
TOTAL A-E	312.4	>971.6	424.8	228.4
F	>1740.0	>1736.8	>605.6	>620.8

9.2.2.5. Summary of results on growth inhibitors

(i) Inhibitor concentrations in leaves 7-12 tend to increase as leaves age at 24°/19°C and decrease at 30°/25°C in both species. The same effect is not always seen in leaves 13-18, and the trend is not clearly established.

(ii) In *E. grandis*, the concentration of inhibitors in young leaves increases with increasing seedling age at both temperatures, but the increase is more marked at 30°/25°C. In *E. regnans* the total concentration of zones A to E decreases as seedlings age at both temperatures, but the concentration of zone F increases as in *E. grandis*.

(iii) In the oldest seedlings studied, inhibitor concentrations decreased up the seedling[1] in *E. regnans* at both temperatures, while in *E. grandis* concentrations increased strongly with increasing height in the seedling at $30^{\circ}/25^{\circ}C$ but showed little variation with position in the seedling at $24^{\circ}/19^{\circ}C$.

(iv) The overall level of inhibitor concentrations in zones A to E is similar in both species at $24^{\circ}/19^{\circ}C$, but an increase in temperature to $30^{\circ}/25^{\circ}C$ leads to more than 3 times as much inhibition in *E. grandis* and only about half as much in *E. regnans*. Inhibitor concentration in zone F is not affected by temperature, but levels in *E. grandis* are about 3 times as high as in *E. regnans*.

9.3. Discussion

The preliminary bioassays for acid inhibitors and gibberellin-like promoters described in Chapter 6 led to the suggestion that in each species there were active substances of both types which showed no distinct response to differences in growing temperature or age, but there was evidence for the presence of at least one inhibitor and one promoter whose concentrations varied considerably between different temperature regimes, species and seedling ages in a way which suggested their concentrations might be correlated with the observed growth rates of the seedlings. The more detailed separation and quantitative assessment of promoting and inhibiting activity at two temperatures described in this Chapter shows that the situation is in fact much more complex.

[1]See footnote to p. 195.

It has been shown that approximately eight promoters and six inhibitors are present in the leaves of both species, and that the concentrations of all these vary with growing temperature and/or leaf and seedling age. No evidence was found for the complete absence from one species of active substances important in the other, although there were several examples of the apparent absence of a substance at one temperature which was active at the other. However, as comparisons between bioassays were based only on activity in specific zones, it is by no means certain that the same substances were always responsible for activity in a particular zone and qualitative differences between the two species may have gone undetected. An attempt was made to characterise the substances responsible for major promoting activity in some zones by mass spectrometry, but while providing further evidence for the gibberellin-like nature of some of these it did not lead to a clear identification. The details of this work are set out in Chapter 10.

The major contribution of the bioassays discussed in this Chapter is the information they provide on the quantitative effects of age and temperature on active compounds present in both species. In spite of the approximate nature of the data, resulting from the limitations of the chromatography-bioassay approach, the simultaneous assay of both inhibitors and promoters, and the lack of replication of the bioassays, they demonstrate a very real reduction in promoting and inhibiting activity in *E. regnans* and increase in inhibiting activity in *E. grandis* at $30^{\circ}/25^{\circ}C$ compared with $24^{\circ}/19^{\circ}C$.

With regard to changes in overall growth substance activity with age (the combination of leaf age and seedling maturity effects), a distinct interaction with growing temperature was found only in the inhibitor concentration data: the large difference between temperature regimes obvious at the 24 leaf-pair stage was completely absent at the 18 leaf-pair stage. The difference in promoter concentrations between temperatures was well developed by the 18 leaf-pair harvest, the earliest age at which temperature comparisons can be made, so there is no evidence for a similar interaction of age and temperature in these data.

An attempt to correlate the major effects of temperature and age on growth substance activity with the observed differences in growth rate and other characteristics of the seedlings leads to perplexing questions as to the role of these substances in the plant. The large decrease in promoter concentrations in *E. regnans* at $30^{\circ}/25^{\circ}$C compared with $24^{\circ}/19^{\circ}$C, while concentrations in *E. grandis* vary only slightly between temperatures, is reasonable in view of the depressed growth of *E. regnans* at the higher temperature. It is difficult however to explain why one species should require concentrations of gibberellin-like promoters so much higher than the other for normal growth at $24^{\circ}/19^{\circ}$C, particularly when the growth rate of *E. grandis* has been shown to be faster than that of *E. regnans* throughout the temperature range from $18^{\circ}/13^{\circ}$C to $33^{\circ}/28^{\circ}$C.

The effect of increased temperature on inhibitor concentrations is more surprising - their reduction in the slower growing species and large increase in concentration in

the species whose growth is accelerated at the higher temperature indicates that the major substances involved are not acting as inhibitors of growth in the plants. Such an increased inhibitor content in rapidly growing material has been reported previously in lilac (von Guttenberg and Leike 1958); also, inhibition in the cress bioassay does not necessarily indicate that the substance responsible is an inhibitor of growth in *Eucalyptus* species. Only in inhibitory zone D, the only zone whose activity increases with seedling age in newly formed leaves of *E. regnans*, is there a correlation of concentration with growth rate. The major inhibitors of zones B, D, E and F in *E. grandis* may be by-products of metabolism stored in the leaves, the increase in concentration of which at $30^{\circ}/25^{\circ}$C is a direct result of the accelerated rate of growth and development at this temperature.

The results obtained from the comparison of methanol and detergent extraction procedures described in the Appendix suggest that the bioassay data discussed in this Chapter may not accurately reflect the differences in total gibberellin content between species. If the enhancement of activity resulting from detergent extraction is indeed due to extraction of gibberellins not extractable by methanol, and if the difference in enhancement between species (3.5 times in *E. regnans*, 12 times in *E. grandis*) persists in leaf samples from different harvests, then the changes in total gibberellin content with age and temperature might differ considerably from those reported here.

This does not necessarily reduce the value of these results: Browning and Saunders (1977) consider it likely that the membrane-bound gibberellins are located in a separate cell compartment from the methanol-extractable gibberellins, and hence are probably involved in different processes. As long as it is realised that the effects of temperature and age discussed in this Chapter refer to methanol-extractable gibberellins, their significance is not decreased by the possible presence of other gibberellins which may or may not show similar responses to age and temperature.

CHAPTER 10

MASS SPECTROMETRY OF GROWTH PROMOTING FRACTIONS FROM LEAF EXTRACTS

10.1. Introduction

The study of growth promoting and inhibiting compounds described in Chapters 8 and 9 dealt with them in terms of 'active zones' in bioassays of chromatographed leaf extracts, without identifying the compounds responsible for the activity. In the preliminary investigations however some evidence for the gibberellin-like nature of at least some of the promoters was obtained from their fluorescence characteristics on TLC plates and in solution. As a great deal of work has been done on mass spectrometric identification of the gibberellins (Binks *et al*. 1969, MacMillan and Pryce 1973, Takahashi *et al*. 1969) it was considered worthwhile to attempt a further characterisation and possibly unequivocal identification of some of the important promoters present using this method. If activity in a particular zone could be attributed to a known gibberellin, the variation in activity in that zone between species and temperature regimes could be compared with what is known of the metabolism of gibberellins in other species.

However, most of the work on structure determination or identification of gibberellins in extracts of plant tissues by mass spectrometry has used large quantities of material (typically 1 kg but often more), commonly from seeds and other tissues known to be rich in gibberellins (Murofushi *et al*. 1968,

Yamaguchi *et al*. 1970). An important exception is the identification of GA_4 and GA_9 in extracts from 10 g samples of wheat leaves by Browning and Saunders (1977), made possible by the use of detergent extraction in place of methanol extraction to release gibberellins from the chloroplast membrane in very much higher concentrations than attainable by the usual extraction methods. Furthermore, since 1967 mass spectrometry has been combined with gas-liquid chromatographic separation methods to enable spectra to be determined on samples of virtually pure substances, even from complex mixtures such as plant extracts. This very powerful technique (GC-MS) has been responsible for many of the successful identifications of gibberellins from plant tissues (MacMillan *et al*. 1967, MacMillan and Pryce 1968, Pryce and MacMillan 1967, Pryce *et al*. 1967).

The quantities of leaf material available in this study were relatively small, but the bioassays detailed in Chapter 9 demonstrated the presence of up to 3 µg/kg GA_3 equivalent in active zones from chromatographic columns and plates. Since few gibberellins are as active as GA_3 in the lettuce hypocotyl bioassay (Crozier *et al*. 1970) it is not unreasonable to expect to obtain 1-2 µg of an active unknown promoter from an extract of 50-100 g of leaves. The strong fluorescence reaction obtained when a few drops of some active fractions are chromatographed by TLC and the plates sprayed and heated as described in Chapter 8 provides encouraging evidence for the presence of gibberellins in reasonably high concentrations in these fractions.

Modern mass spectrometers are capable of determining the spectrum of a compound from a sample as small as 1 µg, but obviously with such a limiting quantity a high degree of purity is necessary or the spectrum of the target compound may easily be obscured or confused by that of a contaminant. As no gas chromatograph was available for this study, the column and thin layer chromatographic methods described in Chapter 8 were relied upon to provide active fractions of sufficient purity for mass spectrometry. It is unlikely however that the fractions obtained were as pure as desirable; further purification of active fractions by repeated TLC in different solvent systems is possible, but would almost certainly involve some loss of the active compound, reducing the chance of obtaining a clear spectrum. In addition, eluates from silica gel TLC plates were considered unsuitable for mass spectrometry by Pitel *et al*. (1971), presumably due to interference from soluble components of the binding agent, while silica gel column fractions were considered satisfactory.

In view of these considerations, it is not surprising that a clearly identifiable gibberellin mass spectrum was not obtained from the very small samples of relatively impure promoters used here, many of which were eluted from silica gel TLC plates. Nevertheless, some fractions did give spectra which show important features characteristic of gibberellin mass spectra, and provide sufficient information to infer certain structural details of the compounds responsible. Thus, while not providing unequivocal identification of gibberellins in the leaf extracts, the mass spectra obtained do provide further evidence that the temperature- and species-dependent growth

promoting activity in the acid fractions of the leaf extracts is caused at least in part by gibberellins.

10.2. Survey of promoting fractions

The acid fractions from 50 g of young leaves from the *E. grandis* 18 leaf-pair harvest and the *E. regnans* 24 leaf-pair harvest of seedlings grown at $30^{\circ}/25^{\circ}C$ were extracted and chromatographed by the methods described in Section 8.3.3. These leaves were chosen as material which had shown significant promoting activity in a wide range of active zones in bioassays. Column fractions were dried on a rotary evaporator and taken up in wet ethyl acetate; TLC plates were divided into ten Rf zones and eluted with wet ethyl acetate. Approximately 0.3 ml of each fraction (equivalent to about 5 g fresh weight of leaves) was spotted on a silica gel G plate and chromatographed with ethyl acetate: chloroform: acetic acid 15:5:1 v/v as solvent. A reference spot of GA_3 was included on each plate.

After drying, the plates were sprayed with 5% H_2SO_4 in ethanol and heated for ten minutes at $120^{\circ}C$. Inspection in ultraviolet light revealed a strong light blue spot at Rf 0.35-0.37 due to GA_3, and spots due to the *E. grandis* fractions as follows: fraction 2 - yellow at Rf 0.35; fraction 16 - pale yellow at Rf 0.12; and fraction 25 - very strong yellow at Rf 0.18. The *E. regnans* fractions developed a pale spot at Rf 0.20 from fraction 16 and a blue-violet spot at Rf 0.60 from fraction 22. The last of these corresponds both in colour of fluorescence and in Rf in water and ethyl acetate: chloro-

form: acetic acid to GA_4 (Elson *et al*. 1964, Cavell *et al*. 1967), while the *E. grandis* fraction 25 spot shows similar chromatographic behaviour in these two solvents to GA_2, which however fluoresces purple on heating. The fractions which produced a distinct colour reaction were considered most likely to contain a sufficient concentration of gibberellin to enable identification by mass spectrometry.

The gibberellins are relatively non-volatile compounds which must be converted to more volatile derivatives, usually the methyl esters or their trimethyl silyl ethers, for mass spectrometry. Fractions selected on the basis of biological activity and TLC fluorescence were methylated by the addition of diazomethane in dichloromethane to the dried fractions, followed by evaporation to dryness in a rotary evaporator. Diazomethane was prepared on a micro scale, basically by the method of Grunwald *et al*. (1967). Three test tubes were connected in series, the first containing 20 ml of 10% methanol in diethyl ether, the second 2 ml of 60% KOH, 2 ml of carbitol and 220 mg of N-methyl N-nitroso-p-toluenesulphonamide (Diazald), and the third 15 ml of dichloromethane. Nitrogen gas was bubbled through the first tube to the second, in which it picked up the diazomethane as it was generated and carried it to the third tube where a yellow colouration indicated the collection of diazomethane in the solvent.

Mass spectra of the methylated fractions, taken up in a small volume of methanol, were obtained by direct insertion into the ionization chamber of a Varian CH7 mass spectrometer

operating at 70 eV at a source temperature of 30°-40°C. Distinct spectra were obtained from most of the fractions examined, although in one case (*E. grandis* fraction 16) little more than the spectrum of the methanol solvent was present. The spectra obtained from column fractions were all very similar up to m/e 300, differing only in the relative intensities of the major ions, which included m/e 43, 57, 74, 87, 149, 239, 270 and 298. Above m/e 300 all the spectra were very weak, with only a few ions of intensity more than 1% of the base peak. In this range however, there were distinct differences between the spectra of different fractions. It would appear that any active compounds in these fractions were not present in high enough concentrations to produce a clear spectrum and that the spectra obtained are dominated by solvent- or column-derived impurities present in all fractions.

The spectra of fractions obtained from TLC differed considerably from those of column fractions: while m/e 43 and 57 were still usually the most intense ions, the intensities of the other ions which characterised the column fractions were much reduced so that except for *E. grandis* fraction 25 the spectra lacked strong ions above m/e 100. Again, it is likely that the concentrations of any active substances present were inadequate to produce a distinct spectrum. Some negative conclusions can be drawn; in particular, the spectrum of *E. regnans* fraction 22 does not contain the ions at m/e 224, 284 or 314 which characterise the mass spectrum of GA_4 methyl ester (Binks *et al.* 1969) and it is therefore unlikely that this fraction contained GA_4, in spite of its similar chromatographic behaviour.

10.3. Mass Spectrum of the zone H Promoter

The mass spectrum of *E. grandis* fraction 25 (Figure 10-1) was distinctly different from all the others, and is worth considering in more detail. The fluorescence reaction obtained from this fraction on TLC plates was particularly strong, and it may be that the concentration of active substance there was rather higher than in other fractions.

The most obvious features of the spectrum are the very strong peaks at m/e 147 (the base peak) and 178, with less intense but also important peaks at 119 and 91, as well as the m/e 43 and 57 peaks seen in the spectra of other fractions. Apart from these there is a distinct lack of prominent peaks especially in the upper range of the spectrum, where gibberellin methyl esters have their strongest peaks due to such ions as M-32, M-60, M-92 and M-122. However, the methyl esters of GA_2, GA_8 and GA_{10} at least have a strong base peak in the lower range and no other strong peaks (Binks *et al.* 1969), so the lack of these does not preclude a gibberellin structure.

Interpretation of mass spectra of gibberellin methyl esters is complicated by the fact that molecular ions are not always apparent; in this spectrum the highest distinct ion is at m/e 364, but it is tempting to propose 366 as an unseen molecular ion. On this basis the upper part of the spectrum would contain ions at M-2 (loss of H_2), M-17 (OH), M-31 (CH_3O), M-44 (CO_2) and M-59 ($COOCH_3$), all of which are typical of gibberellin methyl esters (Binks *et al.* 1969; Takahashi *et al.* 1969; MacMillan and Pryce 1973). However, none of the known gibberellins has a methyl ester of molecular weight 366 and an

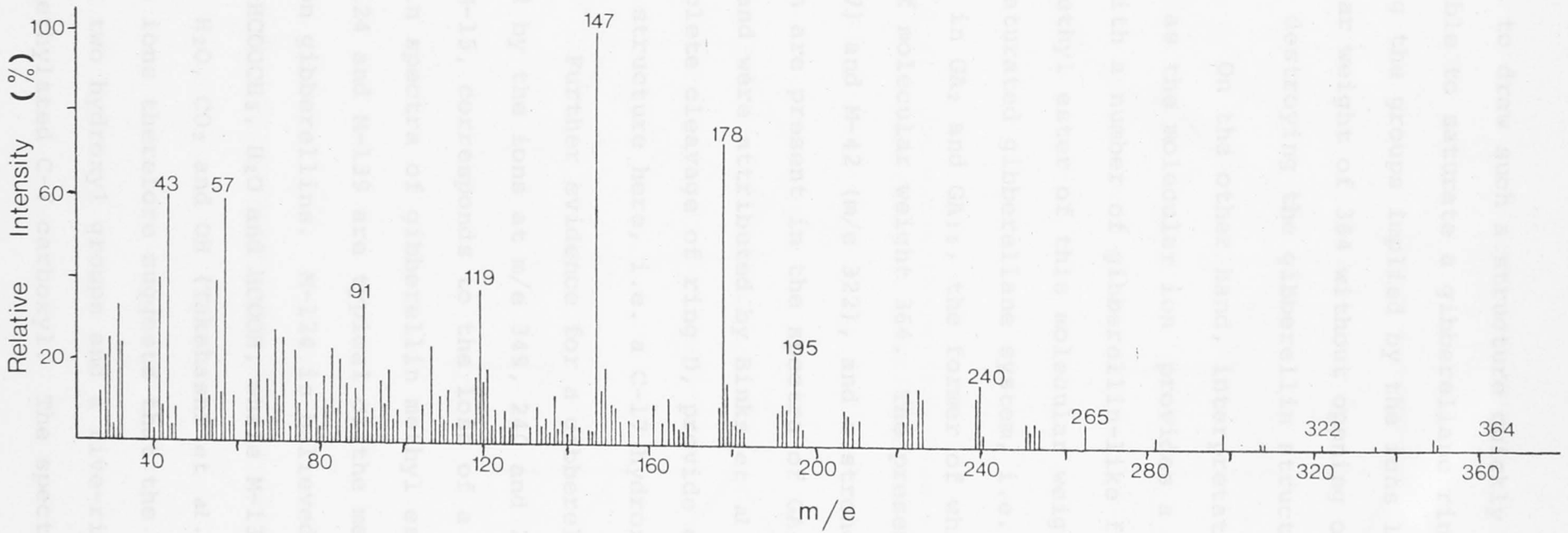


Figure 10-1. Mass spectrum of methylated fraction 25 from leaves of *E. grandis*.

attempt to draw such a structure quickly reveals that it is impossible to saturate a gibberellane ring system (Figure 10-2), carrying the groups implied by the ions listed above beyond a molecular weight of 364 without opening one of the rings and thereby destroying the gibberellin structure.

On the other hand, interpretation of the peak at m/e 364 as the molecular ion provides a plausible spectrum, again with a number of gibberellin-like features. A gibberellin methyl ester of this molecular weight must contain a fully saturated gibberellane system, i.e. carbon 17 is saturated as in GA_2 and GA_{10}, the former of which also has a methyl ester of molecular weight 364. The presence of ions at M-57 (m/e 307) and M-42 (m/e 322), and a strong peak at m/e 43, all of which are present in the spectra of GA_2 and GA_{10} methyl esters and were attributed by Binks *et al*. (1969) to partial and complete cleavage of ring D, provide evidence for a similar structure here, i.e. a C-17 hydroxyl group.

Further evidence for a gibberellin structure is provided by the ions at m/e 349, 240 and 225. The first of these, M-15, corresponds to the loss of a methyl group and is common in spectra of gibberellin methyl esters. The other two, M-124 and M-139 are typical of the methyl esters of many 19-carbon gibberellins. M-124 is believed to result from the loss of $HCOOCH_3$, H_2O and HCOOH, while M-139 is due to loss of $HCOOCH_3$, H_2O, CO_2 and OH (Takahashi *et al*.1969); the presence of these ions therefore suggests that the compound contains at least two hydroxyl groups and a five-ring lactone as well as the methylated C-7 carboxyl. The spectrum also contains

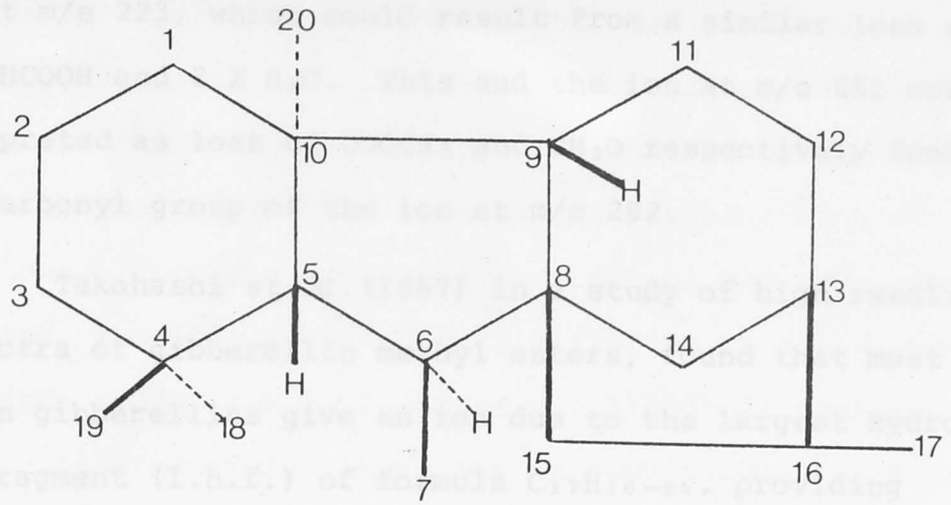


Figure 10-2. The ent-gibberellane ring system (numbering according to McCrindle and Overton, 1965).

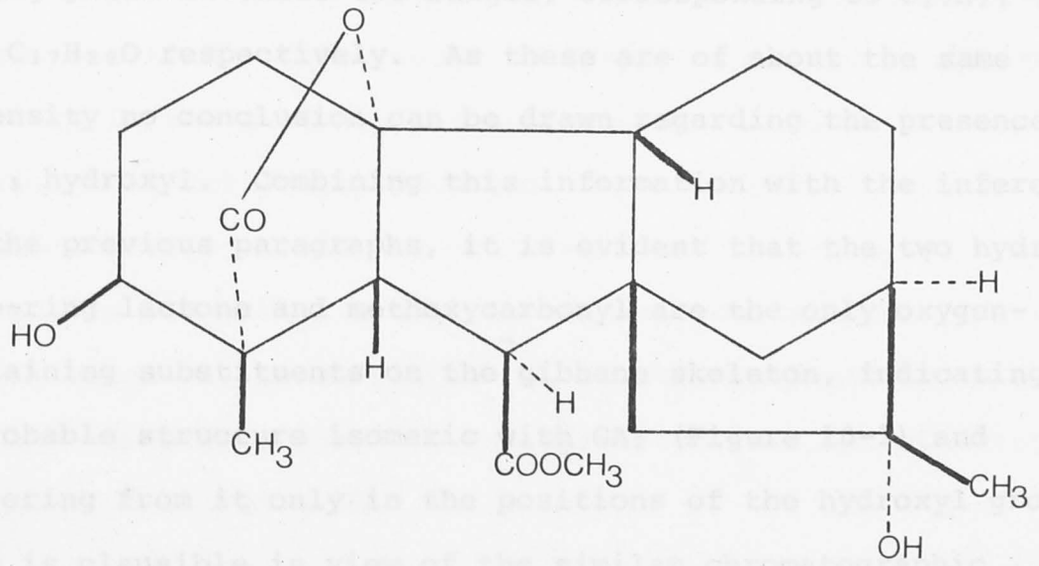


Figure 10-3. Structure of GA_2 methyl ester.

an ion at m/e 223, which could result from a similar loss of $COOCH_3$, HCOOH and 2 X H_2O. This and the ion at m/e 251 could be interpreted as loss of $COOCH_3$ and CH_3O respectively from a methoxycarbonyl group of the ion at m/e 282.

Takahashi *et al.*(1967) in a study of high resolution mass spectra of gibberellin methyl esters, found that most 19-carbon gibberellins give an ion due to the largest hydrocarbon fragment (l.h.f.) of formula $C_{17}H_{16-24}$, providing evidence for the gibbane skeleton. An ion of formula $C_{17}H_{14-25}O$ is also commonly present, and tends to be stronger than the l.h.f. ion when a C-13 hydroxyl group is present. In this spectrum the m/e 225 and 240 ions are the only relatively strong peaks in these two ranges, corresponding to $C_{17}H_{21}$ and $C_{17}H_{20}O$ respectively. As these are of about the same intensity no conclusion can be drawn regarding the presence of a C_{13} hydroxyl. Combining this information with the inference of the previous paragraphs, it is evident that the two hydroxyls, five-ring lactone and methoxycarbonyl are the only oxygen-containing substituents on the gibbane skeleton, indicating a probable structure isomeric with GA_2 (Figure 10-3) and differing from it only in the positions of the hydroxyl groups. This is plausible in view of the similar chromatographic behaviour of the compound.

There are however two important problems with this interpretation of the spectrum. The first of these is the absence of some of the most characteristic ions, particularly M-32 (CH_3OH) and M-60 ($HCOOCH_3$) from the methoxycarbonyl group: these are characteristic of methyl esters in general, and are

present in the spectra of all the methylated gibberellins 1 to 24 published by Binks *et al.*(1969). The M-124 and M-139 peaks suggest that methoxycarbonyl groups are lost in combination with others, yet there is no sign of the fragmentation of these groups alone. This may be simply a result of the very small sample size, possibly combined with different operating conditions from those used by Binks *et al.* (1969) in their GC-MS analysis. Alternatively, there may be some structural feature of the compound, not present in any of GA_{1-24}, which interacts with the methoxycarbonyl group and hinders its removal from the molecular ion.

The second problem is the interpretation of the base peak at m/e 147 and the other strong peaks in the lower part of the spectrum. These could be simply explained as due to an impurity in the extract, perhaps present in greater concentration than the active compound. As a component of the original acid fraction, it could well be a carboxylic acid present as its methyl ester in the analysed sample: if m/e 178 is taken as the molecular ion of this compound the m/e 147 and 119 peaks correspond to the characteristic methyl ester losses of M-31 and M-59.

On the other hand, it is possible that the strong peaks in the m/e 100-200 range are part of the main spectrum, and the presence of these unusual ions may be related to the equally unusual lack of significant methoxycarbonyl losses from the molecular ion. One possible interpretation of the fragmentations involved is shown in Figure 10-4: this does not however indicate the proposed interaction resulting in retention of the methoxycarbonyl and should be seen as no more

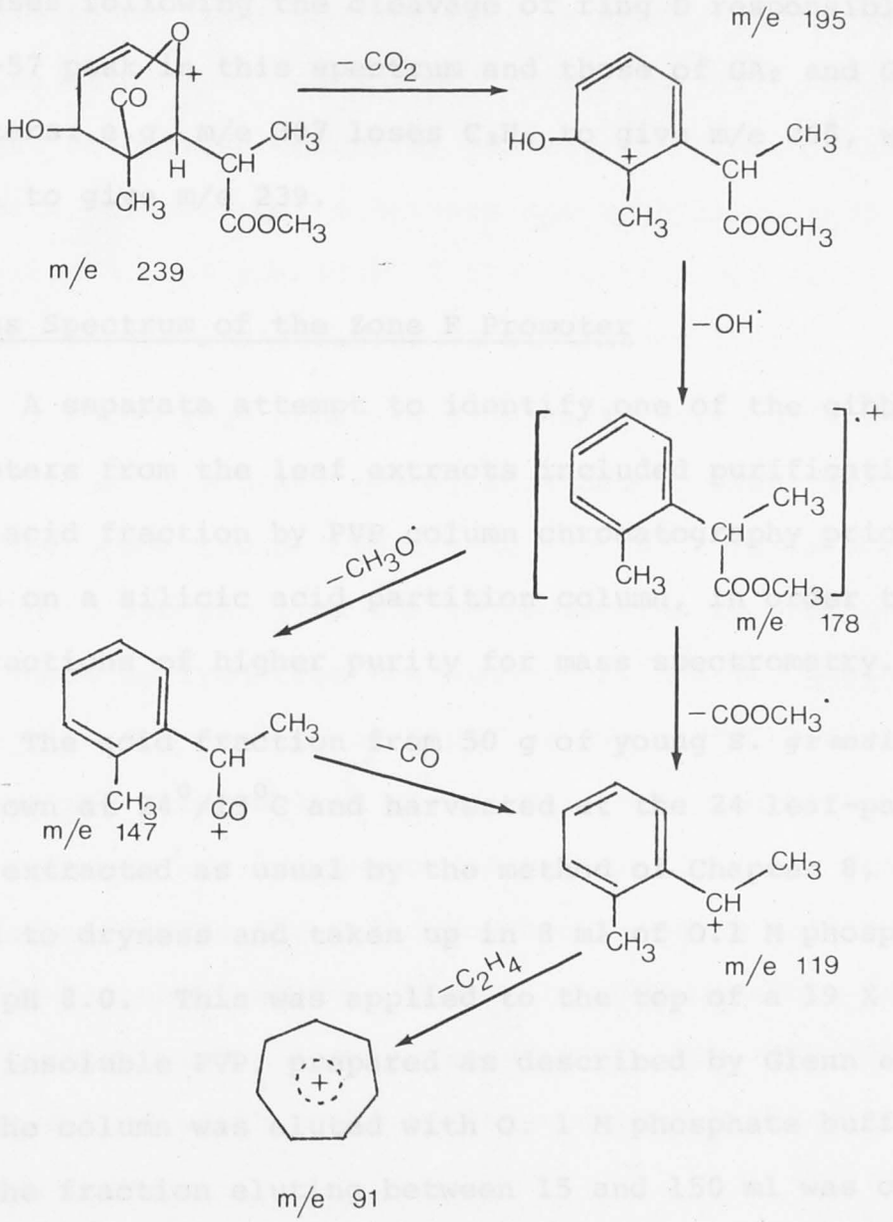


Figure 10-4. Possible fragmentation responsible for some of the ions of Figure 10-1.

than a demonstration that these ions could plausibly arise from a gibberellin structure. The m/e 239 ion shown could result from fragmentation of rings B and C with small hydrocarbon losses following the cleavage of ring D responsible for the M-57 peak in this spectrum and those of GA_2 and GA_{10} methyl esters: e.g. m/e 307 loses C_3H_6 to give m/e 265, which loses C_2H_2 to give m/e 239.

10.4. Mass Spectrum of the Zone F Promoter

A separate attempt to identify one of the gibberellin-like promoters from the leaf extracts included purification of the crude acid fraction by PVP column chromatography prior to separation on a silicic acid partition column, in order to provide fractions of higher purity for mass spectrometry.

The acid fraction from 50 g of young *E. grandis* leaves grown at $24^{\circ}/19^{\circ}$C and harvested at the 24 leaf-pair stage was extracted as usual by the method of Chapter 8, evaporated to dryness and taken up in 8 ml of 0.1 M phosphate buffer at pH 8.0. This was applied to the top of a 19 X 1.6 cm column of insoluble PVP, prepared as described by Glenn *et al.* (1972). The column was eluted with 0. 1 M phosphate buffer pH 8.0, and the fraction eluting between 15 and 150 ml was collected. This cleaned acid fraction was adjusted to pH 3.0 and partitioned three times against equal volumes of ethyl acetate. The combined organic phases were evaporated to dryness then chromatographed by the usual combined silicic acid column-TLC procedure of Section 8.3.3.

Bioassay of part of each fraction, corresponding to 10 g fresh weight of leaves, revealed strong promotion in

fractions 17 to 19, i.e. at Rf 0.1 to 0.4 of the TLC plate. After spraying a strip along the side of the plate with 5% H_2SO_4 in ethanol and heating 10 minutes at 120°C, a blue fluorescence in UV light was apparent at Rf 0.25. The remainder of the plate between Rf 0.2 and 0.3 (fraction 18), corresponding to about 30 g fresh weight of leaves, was eluted with wet ethyl acetate, evaporated to dryness and methylated with diazomethane. A mass spectrum of the fraction was obtained as before by direct insertion at 70°C (Figure 10-5).

The spectrum obtained is clearly quite different from any of those discussed above, including a great deal more detail in the upper range from m/e 300 to 400. It is however still likely to contain ions due to impurities in the sample including those derived from the binders of the TLC plate. Comparison with the spectra of Binks *et al.* (1969) indicates that none of the gibberellins GA_1 to GA_{24} are present in this fraction, although again a number of gibberellin-like features can be seen, including the methoxycarbonyl losses missing from the spectrum discussed above. There are four possible fragmentation series, with molecular ions at m/e 410, 400, 386 and 368, any or all of which could be derived from gibberellins in the original extract.

If m/e 410 is taken as the molecular ion, fragments at M-18 (H_2O), M-31/32 (CH_3OH), and M-59/60 ($HCOOCH_3$) indicate the presence of hydroxyl and methoxycarbonyl groups while those at M-46 (H_2O + CO) and M-87/88 ($HCOOCH_3$ + CO) may result from an aldehyde group, although M-28 is not present. However,

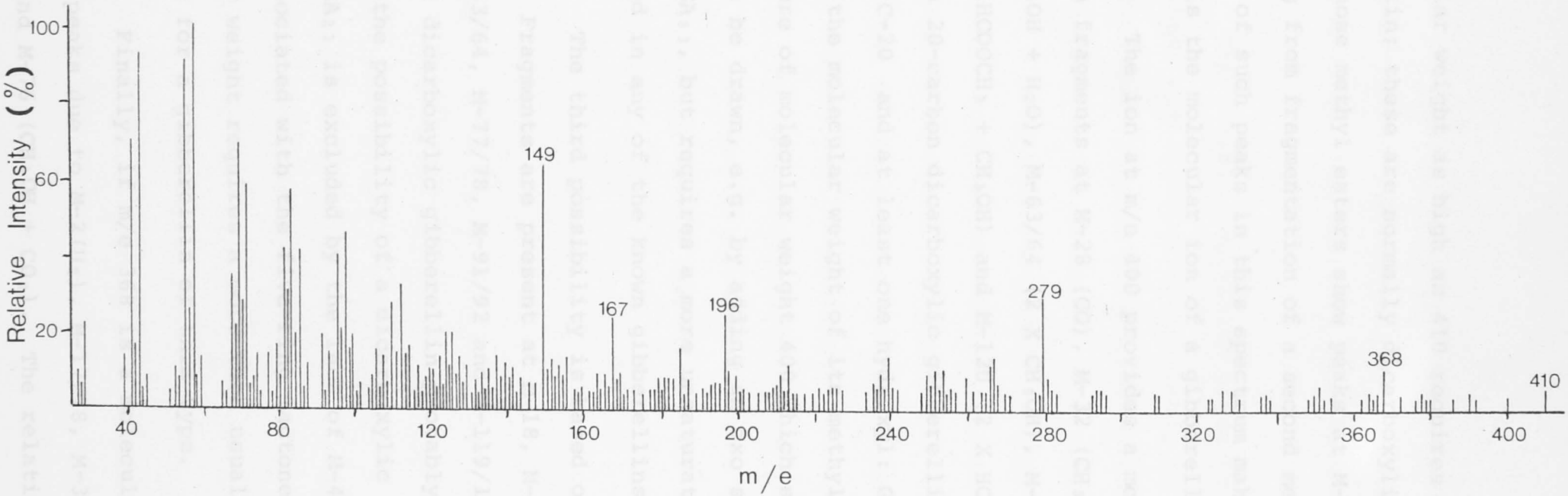


Figure 10-5. Mass spectrum of methylated fraction 18 from leaves of *E. grandis*.

a molecular weight as high as 410 requires a 20-carbon gibberellin; these are normally dicarboxylic or tricarboxylic acids, whose methyl esters show peaks at M-91/92 and M-119/120 resulting from fragmentation of a second methoxycarbonyl group. The lack of such peaks in this spectrum makes it unlikely that m/e 410 is the molecular ion of a gibberellin methyl ester.

The ion at m/e 400 provides a more likely molecular ion, with fragments at M-28 (CO), M-32 (CH_3OH), M-46 (CO + H_2O), M-50 (CH_3OH + H_2O), M-63/64 (2 X CH_3OH), M-78 ($HCOOCH_3$ + H_2O), M-91/92 ($HCOOCH_3$ + CH_3OH) and M-120 (2 X $HCOOCH_3$). These suggest a 20-carbon dicarboxylic gibberellin with an aldehyde group at C-20 and at least one hydroxyl: GA_{19} has this structure but the molecular weight of its methyl ester is only 390. A structure of molecular weight 400 which satisfies the requirements can be drawn, e.g. by adding an oxo substituent as in GA_{26} or GA_{33}, but requires a more unsaturated ring system than that found in any of the known gibberellins.

The third possibility is based on a molecular ion at m/e 386. Fragments are present at M-18, M-32, M-36 (2 X H_2O), M-60, M-63/64, M-77/78, M-91/92 and M-119/120, suggesting a 20-carbon dicarboxylic gibberellin probably with a methyl group at C-20; the possibility of a dicarboxylic 19-carbon gibberellin such as GA_{21} is excluded by the lack of M-44, M-46, M-104 and M-106 associated with the five-ring lactone. Again, the molecular weight requires a more than usually unsaturated structure for a gibberellin of this type.

Finally, if m/e 368 is a molecular ion the spectrum contains peaks due to M-2(H_2), M-17/18, M-31/32, M-46 (HCOOH), M-59/60 and M-76 (CH_3OH + CO_2). The relatively low molecular

weight and presence of M-46 (probably from a five-ring lactone) suggest a 19-carbon gibberellin, but this would require some substituent other than hydroxyl to make a molecular weight of 368.

The overall impression given by this spectrum is that at least one gibberellin is likely to be present and hence responsible for the growth promoting and TLC fluorescence characteristics of the fraction. The spectrum obviously is confused as a result of impurities in the extract, so that definite inferences concerning the structure of the active compound cannot be drawn. However, none of the gibberellins A_1 to A_{24} can be responsible and from a consideration of molecular weights and likely molecular ions, it is unlikely that any of the other known gibberellins would give such a spectrum; it is therefore possible that a new gibberellin or gibberellin derivative is responsible for the promoting activity of the fraction.

CHAPTER 11

DISCUSSION AND CONCLUSIONS

11.1. Introduction

In Chapter 5 evidence was presented for the possible involvement of growth regulating substances in the effects of temperature on growth and morphology of *E. regnans* and *E. grandis* seedlings, and it was suggested that differences between the two species in the growth substances normally present, or in their stability at elevated temperatures, might play a role in causing the observed difference in optimum temperatures. Consequently, an investigation of growth substance concentrations in eucalypt seedlings was undertaken and the results of these studies are described in Chapters 6 to 10.

It is convenient to discuss the possible relations between the effects of temperature on growth substance concentrations and effects on seedling morphology in terms of the elementary model shown in Figure 11-1. This is basically similar to Levitt's (1972) classification of primary stress injuries, without the distinction between reversible (elastic) and irreversible (plastic) effects. A stress, in this case high temperature, is perceived by receptors in the plant. Possible mechanisms of perception include effects on enzyme structure, changes in reaction rates and equilibria, and destruction of labile complexes. If the receptor is such that the temperature-induced change in it directly affects a measurable character or process of the plant, a direct stress effect results; an example is the reduction of photosynthesis

due to the denaturation of a key photosynthetic enzyme. On the other hand, if perception of the stress by the receptor causes a change in a mediator, for example the concentration of another substance or the rate of another reaction, which then affects some measurable character or process, the observed stress effect is indirect.

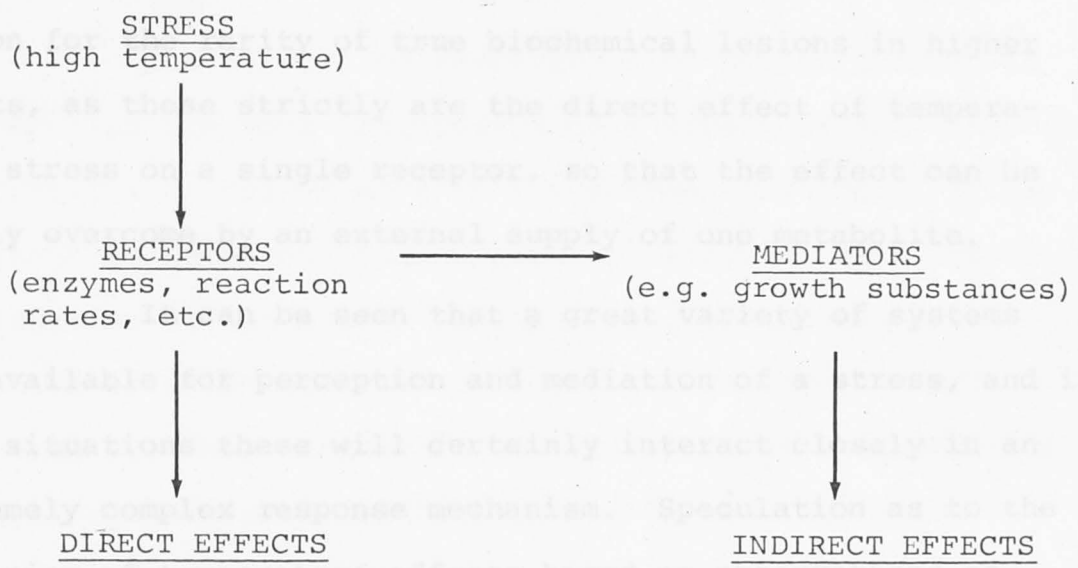


Figure 11-1. A simple model of the elements involved in responses of plants to an external stress.

Due to the intricate nature of biochemical systems, in which the products of each reaction may regulate and be regulated by several others, the distinction between direct and indirect stress effects is not sharp in practice. A direct stress effect may also act as mediator for a number of indirect effects; e.g. denaturation of a single enzyme involved in lateral transport of photosynthate into the phloem stream in the leaves may result in a low sugar concentration in the sap; but this may mediate an indirect starvation effect

in the roots. Clearly the effects involved are more complex than suggested by Figure 11-1. In addition, it seems likely that at any given degree of stress, more than one of the innumerable systems which could behave as receptors will perceive the stress. These may cause different effects, or reinforce each other in causing the same effect; this simultaneous perception by several plant systems is probably the reason for the rarity of true biochemical lesions in higher plants, as these strictly are the direct effect of temperature stress on a single receptor, so that the effect can be wholly overcome by an external supply of one metabolite.

It can be seen that a great variety of systems are available for perception and mediation of a stress, and in real situations these will certainly interact closely in an extremely complex response mechanism. Speculation as to the mechanism of temperature effects based on observations of changes in a small number of plant characteristics can therefore only suggest gross over-simplifications of the actual mechanism; nonetheless, by correlating morphological and physiological effects with changes in the concentration of specific substances an understanding of some of the processes involved in stress response may eventually be gained.

11.2. Direct effects of high temperature

It is difficult to see how any of the effects of temperature on growth and morphology of *E. grandis* and *E. regnans* seedlings could occur directly. The processes constituting growth and differentiation, and their regulation, are too complex to be altered greatly by such a one-step

process. Perhaps the most likely direct effect would be through changes in the concentrations of growth substances resulting from a shift in the position of equilibrium (the receptor) between their active and inactive forms, e.g. free and bound, or free acid and glucoside. However, growth substances seem more likely to be important as mediators than receptors, and will be discussed as such in the next section.

The effects of temperature on photosynthesis in both species are more suggestive of a direct effect, through modification of one or more of the enzymes involved. It is important to note that denaturation of enzymes, with resulting inactivation of the system they control, is not the only mechanism by which moderate temperatures can have an effect on enzyme systems. Kapoor *et al.* (1976) found that growth at elevated temperatures led to a change in the regulating properties of pyruvate kinase in *Neurospora*. The same type of conformational changes which occur in denaturation may apparently occur to a lesser degree in some enzymes such that activity is retained but substrate affinities and other properties are changed. Nevertheless, some enzymes probably are wholly deactivated even by moderate temperatures; for example Cantliffe (1972) considered nitrate accumulation in spinach at $30^{o}C$ to result from the deactivation of nitrate reductase.

A difference between species in the occurrence or intensity of a direct stress effect can only be due to a difference in the receptors present in each. Thus if the lower optimum temperature for photosynthesis and more rapid decline in CO_2 uptake at supra-optimal temperatures in

E. regnans seedlings is a direct effect, the two species must differ in one or more of the enzymes controlling photosynthesis. A species difference in the isozymes of key enzymes present is plausible, since the difference in intra-molecular bonding could certainly account for a difference in thermal stability.

Such a difference between species from differing thermal environments could readily arise through the mechanisms of natural selection, the genes for relatively labile isozymes reaching vanishingly low frequencies in the high temperature population through early failure of seedlings which inherit them. This implies that variation in thermotolerance of *E. regnans* seedlings should exist, and in fact such variation was clearly present even in the very limited population of seedlings raised for the growth study. A comparison of the subunit composition of important photosynthetic enzymes of this species from clones exhibiting high and low thermotolerance might reveal a major cause of differences in the preferred temperature range for growth of different species.

11.3. Indirect effects of high temperature

The remaining effects of high temperature, that is the majority of the temperature responses observed, are probably indirect effects. In this case, a species difference may be due to a difference in either the receptor or the mediator; as a chain of several mediators may be involved, this approach does not help to identify the likely source of differences between species, as was seen to be possible with direct effects.

Perhaps the most important indirect effect which has been found in this study is the reduction in root respiration rates and respiratory quotients at high temperatures. As discussed in Chapters 3 and 5, this may be an effect of the same receptor which appears to directly reduce photosynthesis at supra-optimal temperatures; it resembles a localised starvation injury, and its confinement to the roots may be because they are remote from the photosynthetic tissues of the plant or because transport processes are (directly) restricted by supra-optimal temperature. Either way, starvation does appear to be a major factor in the failure of *E. regnans* at $30^{\circ}/25^{\circ}C$ and $33^{\circ}/28^{\circ}C$ and the reduced growth of *E. grandis* at $33^{\circ}/28^{\circ}C$.

The variations observed in growth substance concentrations with temperature are also significant. These compounds have great potential as mediators. There is no evidence of their ability to act as receptors, although the possibility of equilibrium effects, as described above, cannot be wholly dismissed. The most obvious receptors associated with these mediators are the enzymes regulating their synthesis and metabolic degradation or de-activation, but retardation of transport processes at supra-optimal temperatures may also cause hormone-mediated indirect effects on growth and differentiation.

Variations in the concentrations of auxin, gibberellin-like promoters and acid inhibitors with temperature have been demonstrated, as have considerable differences between species, both in the promoters and inhibitors present

under favourable conditions ($24^{o}/19^{o}C$) and in the effects on them of a rise in temperature (to $30^{o}/25^{o}C$). It is not surprising that clear correlations between temperature effects on growth substance concentrations and on seedling morphology were not found: the complexity of interactions between these substances is now widely recognised (Leopold and Kriedemann 1975), and it appears that changes in the relative concentrations of several substances rather than the individual absolute levels are more often the cause of effects on growth and other characteristics. Substances which were at first believed to be uninvolved in the regulation of some processes because their concentrations were not correlated with observed effects, have later been found to be important as parts of a multi-component regulating system (e.g. Blake 1973).

As effects of temperature on all the growth substances examined have been found, and shown to be complex in the case of acid promoters and inhibitors where a rise in temperature has different effects on the concentrations of individual active compounds, a change in the balance of regulating substances is certain to occur. That such a complex change does not bring about a chaotic disruption of metabolism and growth in both species is an indication of the adaptability and intricacy of the regulating systems of the plant.

A part of the shifting balance is seen in the relative concentrations of promoters and inhibitors in leaves discussed in Chapter 9. Excluding the zone F inhibitor, whose concentration does not change with temperature, the inhibitor:

promoter ratio increases by a factor of 3 in *E. grandis* with an increase in temperature from 24°/19°C to 30°/25°C, while that in *E. regnans* (lower than *E. grandis* even at 24°/19°C) is relatively unaffected. It may be that the increased inhibitor content of *E. grandis* at high temperatures is a protective mechanism which prevents damage to otherwise thermolabile enzymes (perhaps the photosynthetic enzymes discussed above) at 30°/25°C, allowing the plant to benefit from the increase in photosynthesis at high temperatures.

Cytokinin concentrations have not been examined in detail because no clear difference in the effects of temperature on them between species was found in the preliminary assays described in Chapter 6. However, a comparison of the bioassay results of Chapter 9 with the corresponding assays of Chapter 6 reveals that the preliminary screening procedure only detected a small part of the extensive variation in individual promoter and inhibitor concentrations with temperature. Similarly, important species differences in the effects of temperature on cytokinin levels may have been missed. As discussed in Chapter 5, some of the morphological effects of high temperature on the eucalypt seedlings resemble effects attributed to cytokinins, alone or in combination with other hormones. These include the reduction of apical control and apical dominance, and changes in leaf size, shape and colour. The possibility of cytokinins mediating these effects becomes more plausible in the light of the indications that lateral transport processes in the seedlings may be slowed by supra-optimal temperatures: supply of cytokinins from the roots might well be reduced, thus causing the observed leaf and

branch effects. Even without a direct temperature effect on transport, supply might be disrupted by the reduction in root respiration rates resulting from diminished supply of photosynthate. This could apply equally well to root-synthesised gibberellins, and bring about the observed reduction in promoter concentrations at $30^{\circ}/25^{\circ}$C in *E.regnans* leaves.

11.4. Changes in optimum temperature with age

Clearly, the difference in performance of *E. regnans* and *E. grandis* seedlings in a warm temperate environment is a result of the declining optimum temperature of the former species: if the early optimum was maintained as it is in *E. grandis* , the two species would be equally successful on sites such as that of the Coffs Harbour species trial. Three mechanisms can be postulated as the basis of the declining optimum: exhaustion of reserves, seedling maturity, and seasonal temperature effects. These are discussed in the following paragraphs.

11.4.1. Exhaustion of reserves

Reference has already been made to the probability of a root starvation effect occurring in both species at $33^{\circ}/28^{\circ}$C and in *E. regnans* at lower temperatures. Levitt (1972) described starvation injury as the slowest kind of indirect injury (Chapter 5), and it is possible that this mechanism alone could account for the apparent reduction in the optimum temperature for *E. regnans* with time. In fact, if this was so the true optimum temperature could be around

21°C from an early stage, since although seedlings at higher temperatures grow faster for a time, they could only do so by continually drawing on their reserve foods so that eventual injury is inevitable. The higher photosynthetic optimum for *E. grandis* avoids such an injury at temperatures up to 30°/25°C.

The rate of development of such an injury may accelerate with time, through a decrease in net photosynthesis with seedling age either due to a positive feedback effect from the altered root metabolism, or simply to increasing seedling maturity, as discussed below. Alternatively, an increase in leaf respiration or further obstruction of transport to the roots from either of these causes might bring about such an acceleration.

11.4.2. Seedling maturity

Many species including most of the eucalypts show a change in morphological and physiological characteristics with age such that they pass through a more or less distinct juvenile phase of variable length before reaching maturity. The declining optimum temperature in *E. regnans* seedlings could be seen as an expression of this effect. The best evidence supporting this theory comes from ecological considerations: in the usual environment of *E. regnans* , the ability to make rapid initial growth at high temperatures (on open sites, e.g. after a fire) would confer an advantage, while in older seedlings a lower optimum might be a better adaptation

to the prevailing climate. This is similar to the advantage conferred on juvenile beech seedlings by the production of shade leaves only, as early growth of this species is typically under a canopy (Leopold and Kriedemann 1975); mature plants are adapted to a higher light intensity, and are able to produce sun leaves. *E. grandis*, adapted to a warm climate, maintains a high optimum temperature into maturity.

11.4.3. Seasonal temperature effects

It was pointed out in Chapter 1 that *E. regnans* is native to areas where winter temperatures are usually too low for growth, so that a period of rest occurs. Ashton (1975) described a typical example of this phenomenon, on a site where the growing season lasted 7-9½ months. It is reasonable to suggest that the species might be adapted to such an environment to the extent that a period of rest is essential for continued healthy growth. Cremer (1975) found a very consistent rate of development in terms of the number of leaf-pairs differentiated by *E. regnans* in the field: 9 to 11 pairs of internodes were present in each annual shoot, over a wide range of growth rates. This supports the theory that after a definite amount of seasonal growth, this species requires a period of rest.

In the phytotron, growth is forced to continue by the unchanging temperature regimes, most rapidly at the highest temperatures. This might lead to failure of the seedlings even at lower temperatures after a time, but the observed

result would be a declining optimum temperature since the fastest growing seedlings would be expected to fail first. This hypothesis could readily be tested by applying a period of cold conditions to *E. regnans* seedlings grown for say 16 weeks at $30^{\circ}/25^{\circ}$C, then returning them to the high temperature regime and observing whether the symptoms of supra-optimal temperature stress described in Chapter 2 are developed.

This mechanism is probably related to that of starvation injury discussed above: the adaptation of *E. regnans* to its natural environment may consist of a deliberate exhaustion of reserves during the growing season, with their replacement during the following winter. An ability to maintain relatively high rates of photosynthesis at low temperatures would be important in the replacement of reserves, and there are indications (Chapter 3) that *E. regnans* has such an ability (more so than *E. grandis*, at least).

11.5. Conclusions

Although some of the findings of this project (notably the studies of growth-substance concentrations) have revealed a complexity beyond that anticipated for the mechanisms of high-temperature effects on eucalypts, the objectives stated in Chapter 1 have been satisfied: temperature has been established as a factor capable of causing the difference in performance of eucalypt species at Coffs Harbour, and some insight into the underlying physiological effects has been gained. The conclusions reached may be stated as follows:-

(i) Growth of *E. regnans* at temperatures of $30^{\circ}/25^{\circ}$C and above induces morphological effects resembling

those displayed by cool temperate species at Coffs Harbour, and similar effects may occur at lower temperatures after long exposure. *E. grandis* seedlings display some of the same effects at 33°/28°C, but are not affected as severely as *E. regnans* nor does the optimum temperature appear to decline with seedling age.

(ii) Photosynthesis of *E. grandis* leaves has a higher optimum temperature and is affected less by supra-optimal and more by sub-optimal temperatures, than that of *E. regnans* leaves. This may be partly a result of differences in the isoenzymes of the photosynthetic system in each species.

(iii) The effects of supra-optimal temperature on both species may be due in part to root starvation injury resulting from effects of temperature on photosynthesis, respiration and lateral transport processes.

(iv) Growth substance concentrations in the leaves of the two species are markedly affected by growing temperature, and are probably involved in causing the morphological and other effects observed, but their individual roles are not clear and a complex interaction of several compounds is most likely involved.

(v) The decline in the apparent optimum temperature for growth of *E. regnans* at Coffs Harbour and in phytotron studies may result from the inability of the seedlings to replace exhausted food reserves under conditions of enforced continuous growth.

APPENDIX

EXTRACTION OF MEMBRANE-BOUND GIBBERELLINS FROM CHLOROPLASTS

Although methanol extraction by techniques similar to that described in Section 8.3.2. has been the usual first step in isolation of gibberellins by most workers (e.g. Dunberg 1974; Frydman & MacMillan 1973; Durley *et al.* 1975), recent studies by Browning and Saunders (1977) have cast serious doubts on the ability of methanol to extract membrane-bound gibberellins from chloroplasts. Working with isolated chloroplasts from wheat leaves, they demonstrated that detergent extracts contained up to 1000 times the gibberellin activity of methanol extracts, and that treatment of the chloroplasts with methanol apparently induced irreversible binding of the gibberellins to the membrane protein, preventing subsequent extraction with detergent. Separation of the chloroplast extracts by paper chromatography showed that not all the extractable gibberellins were bound in this way, some being extracted as well by methanol as by detergent; only the relatively non-polar gibberellins, in particular GA_4 and GA_9, showed a marked increase in activity when extracted with detergent.

If this membrane binding effect occurs in the leaves of eucalypts, the use of methanol extraction prior to estimation of gibberellin concentrations may well lead to false conclusions as to the effects of age and temperature on promoting activity. The methods of Browning and Saunders (1977) were therefore applied to the extraction and purification of gibberellins from a sample of leaves from each of *E. regnans*

and *E. grandis*, for comparison with the concentrations estimated from methanol extracts separated by the silica gel partition column / thin layer chromatography procedure described in Section 8.3. The material used for this experiment was sampled from the 7-12 leaves of seedlings grown at $24^{o}/19^{o}C$, harvested at the 24 leaf-pair stage.

10 g of shredded leaf material was placed in a chilled mortar lined with 60 µm mesh nylon cloth and ground for 3 minutes at $2^{o}C$ in 100 ml of an extraction medium modified from that used by Honda (1974), containing 9% sucrose, 25 mM tricine buffer, 8 mM 2-mercaptoethanol, 5 mM magnesium acetate, 32.5 $mg.ml^{-1}$ dextran (M.W. approximately 40,000), 25 $mg.ml^{-1}$ Ficoll and 1 $mg.ml^{-1}$ bovine serum albumin at pH 7.2. The mesh was squeezed dry and the material not retained by it centrifuged at 1000 g for 10 minutes; the supernatant was discarded and the pellet, consisting mainly of intact and broken chloroplasts, was resuspended in 50 ml of 2% Triton X-100 detergent in 25 mM tricine buffer at pH 8.0. As the short grinding was insufficient to release all the chloroplasts from the leaf material, the total chlorophyll content of the extract was estimated at this stage by the method of Arnon (1949) for comparison with the total chlorophyll content of leaf samples from the same harvests, extracted by homogenising in acetone and estimated by the same method.

The chloroplast suspension, diluted to 200 ml with water, was adjusted to pH 8.0 and passed through a 1.6 X 15 cm column of 100 mesh Dowex 1 X4 anion exchange resin, previously washed with 1 M potassium formate of pH 8.0 until the eluate

was free of chloride ions. The loaded column was washed with 200 ml of water adjusted to pH 8.0 to remove the non-ionic detergent, then the gibberellins were eluted with 400 ml of ethanol : 1 M formic acid 4:1 v/v. The eluate was evaporated to a small volume under vacuum at 35°C, diluted with 100 ml 0.1 M Na_2HPO_4 and adjusted to pH 8.0, then stirred for 1 hour with 10 g of insoluble polyvinylpyrrolidone (PVP).

The filtered extract was adjusted to pH 3.0 and partitioned three times against 0.5 volume of ethyl acetate. The acid fraction obtained was dried with anhydrous magnesium sulphate, evaporated to dryness under vacuum at 35°C and the residue taken up in a small volume of methanol. This was applied as a 10 cm strip to a sheet of Whatman no. 1 chromatography paper, developed in the descending direction with isopropanol : 25% ammonia : water 10:1:1 as solvent. After drying, the chromatogram was cut into ten Rf segments and bioassayed directly by the lettuce hypocotyl assay (Frankland and Wareing 1960), using 10 lettuce germinates in 5 ml water for each segment in 9 cm Petri dishes.

The bioassay results for each species are shown in Figure A-1, and may be compared with bioassay data for methanol extracts of leaves from the same harvests in Figure 9-1. It is clear that enhancement of promoting activity of the order reported by Browning and Saunders (1977) in wheat leaves has not occurred here. However, if the regression equation of Figure 8-4 is used to estimate the equivalent concentration of GA_3 in each of the bioassay fractions showing significant promoting activity, a total of 12.55 µg GA_3 equivalent. g^{-1} chlorophyll is found in the *E. regnans* detergent extract

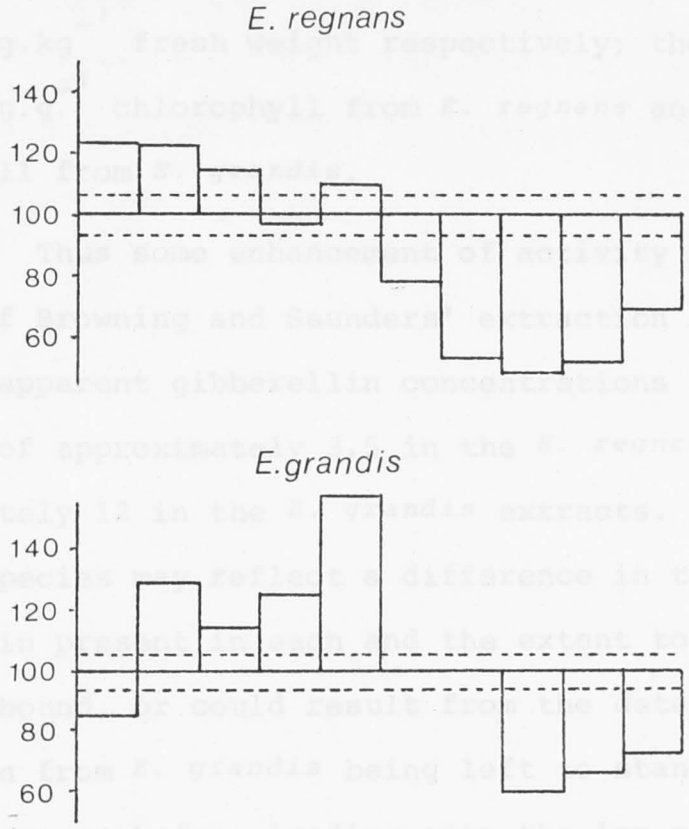


Figure A-1. Lettuce hypocotyl bioassay of detergent-extracted acid fractions after paper chromatography in isopropanol:ammonia:water 10:1:1. Abscissa: Rf 0.1-1.0 from chromatogram; Ordinate: percentage of control hypocotyl length. Dashed lines show 95% confidence limits for the control mean.

and 26.45 $\mu g.g^{-1}$ in that from *E. grandis*. The total GA_3 equivalent concentrations in methanol extracts of leaves from these two harvests as shown in Tables 9-1 and 9-2 are 10.68 and 5.76$\mu g.kg^{-1}$ fresh weight respectively; these correspond to 3.58 $\mu g.g^{-1}$ chlorophyll from *E. regnans* and 2.19 $\mu g.g^{-1}$ chlorophyll from *E. grandis*.

Thus some enhancement of activity has resulted from the use of Browning and Saunders' extraction and purification methods: apparent gibberellin concentrations are increased by a factor of approximately 3.5 in the *E. regnans* extracts and approximately 12 in the *E. grandis* extracts. The difference between species may reflect a difference in the types of gibberellin present in each and the extent to which these are membrane-bound, or could result from the detergent chloroplast suspension from *E. grandis* being left to stand longer than that from *E. regnans* before loading onto the ion exchange column.

It is important to realise that enhancement of gibberellin activity of the order achieved here is known to be attainable by passage of a crude extract through a column of PVP to remove phenolics and other inhibitors of gibberellin activity (Glenn *et al.* 1972; Dunberg 1974), and conceivably the increase in apparent concentration found here is due to the PVP purification step or possibly even the ion exchange column, rather than the use of detergent extraction. As the acid fractions in this study were to be assayed for both promoters and inhibitors, it was considered desirable to attempt to separate the two types of compound chromatographically rather than enhance the activity of one at the expense of the

other: the relatively low levels of gibberellin activity which result from such an approach are considered acceptable for comparisons between different batches of leaves. On this basis, it appears that detergent extraction by the method of Browning and Saunders (1977) was not warranted for the work reported here.

LIST OF REFERENCES

Addicott, F.T., and R.S. Lynch, 1951. Acceleration and retardation of abscission by indoleacetic acid. Science, 114: 688-689.

Andres, J., and H. Smith, 1976. Evidence for a rapid effect of abscisic acid on amino acid metabolism in *Lemna*. Plant Sci. Lett., 6: 315-318.

Anon., 1966. "Rainfall Statistics, Australia". Commonwealth of Australia, Bureau of Meteorology.

Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. Plant Physiol., 24: 1-15.

Ashton, D.H., 1975. The seasonal growth of *Eucalyptus regnans* F. Muell. Aust. J. Bot., 23: 239-252.

Audus, L.J., 1972. "Plant Growth Substances". Vol. 1: Chemistry and Physiology. 533 pp. 3rd ed. Leonard Hill, London.

Bachelard, E.P., 1969a. Effects of gibberellic acid on internode growth and starch contents of *Eucalyptus camaldulensis* seedlings. New Phytol., 68: 1017-1022.

Bachelard, E.P., 1969b. Studies on the formation of epicormic shoots on eucalypt stem segments. Aust. J. Biol. Sci., 22: 1291-1296.

Bell, D.J., 1955. Mono- and oligosaccharides and acidic monosaccharide derivatives. pp. 1-54 in K. Paech and M.V. Tracey (eds.) "Modern Methods of Plant Analysis", Vol. 2, 626 pp. Springer-Verlag, Berlin.

Biddington, N.L., and T.H. Thomas, 1973. A modified *Amaranthus* betacyanin bioassay for the rapid determination of cytokinins in plant extracts. Planta, 111: 183-186.

Bigot, C., 1968. Action d'adénines substituées sur la synthèse des bétacyanines dans la plantule d'*Amarantus caudathus* L. Possibilité d'un test biologique de dosage des cytokinines. Comptes Rendus, Acad. Sci. Paris (series D), 266: 349-352.

Binks, R., J. MacMillan, and R.J. Pryce, 1969. Combined gas chromatography-mass spectrometry of the methyl esters of gibberellins A_1 to A_{24} and their trimethyl silyl ethers. Phytochemistry, 8: 271-284.

Blake, T.J., 1972. Studies on the lignotubers of *Eucalyptus obliqua* L'Herit. III. The effects of seasonal and nutritional factors on dormant bud development. New Phytol., 71: 327-334.

Blake, T.J., 1973. Aspects of apical dominance in *Eucalyptus obliqua* L'Herit. Ph.D. Thesis, University of Melbourne.

Blake, T.J., 1976. Thermodormancy in seedlings of *Eucalyptus obliqua* L'Herit. Aust. J. Plant Physiol., 3: 269-273.

Blake, T.J., and B. B. Carrodus, 1969. Studies on the lignotubers of *Eucalyptus obliqua* L'Herit. II. Endogenous inhibitor levels correlated with apical dominance. New Phytol., 69: 1073-1079.

Brown, A., and N. Hall (eds.),1968. "Growing trees on Australian farms". 397 pp. Department of National Development, Forestry and Timber Bureau, Canberra.

Brown, C.L., R.G. McAlpine, and P.P. Kormanik, 1967. Apical dominance and form in woody plants: a reappraisal. Am. J. Bot., 54: 153-162.

Browning, G., and P.F. Saunders, 1977. Membrane localised gibberellins A_9 and A_4 in wheat chloroplasts. Nature, 265: 375-377.

Cantliffe, D.J., 1972. Nitrate accumulation in spinach grown at different temperatures. J. Am. Soc. Hort. Sci., 97: 674-676.

Cavell, B.D., J. MacMillan, R.J. Pryce, and A.C. Sheppard, 1967. Plant hormones. V. Thin-layer and gas chromatography of gibberellins; direct identification of the gibberellins in a crude plant extract by gas-liquid chromatography. Phytochemistry, 6: 867-874.

Cremer, K.W., 1975. Temperature and other climatic influences on shoot development and growth of *Eucalyptus regnans*. Aust. J. Bot., 23: 27-44.

Crow, W.P., T. Osawa, D.M. Paton, and R.R. Willing, 1977. Structure of grandinol: a novel root inhibitor from *Eucalyptus grandis*. Tetrahedron Lett., 12: 1073-1074.

Crozier, A., H. Aoki, and R. Pharis, 1969. Efficiency of countercurrent distribution, Sephadex G-10, and silicic acid partition chromatography in the purification and separation of gibberellin-like substances from plant tissue. J. Exp. Bot., 20: 786-795.

Crozier, A., C.C. Kuo, R.C. Durley, and R.P. Pharis, 1970. The biological activities of 26 gibberellins in nine plant bioassays. Can. J. Bot., 48: 867-877.

Dunberg, A., 1974. Occurrence of gibberellin-like substances in Norway spruce (*Picea abies* (L.) Karst.) and their possible relation to growth and flowering. Studia Forestalia Suecica, 111. 62 pp.

Durley, R.C., A. Crozier, R.P. Pharis, and G.E. McLaughlin, 1972. Chromatography of 33 gibberellins on a gradient eluted silica gel partition column. Phytochemistry, 11: 3029-3033.

Durley, R.C., and R.P. Pharis, 1972. Partition coefficients of 27 gibberellins. Phytochemistry, 11: 317-326.

Durley, R.C., R.P. Pharis, and J.A.D. Zeevart, 1975. Metabolism of (^{3}H)-gibberellin A_{20} by plants of *Bryophyllum daigremontianum* under long- and short-day conditions. Planta, 126: 139-149.

Durley, R.C., I.D. Railton, and R.P. Pharis, 1973. Interconversion of gibberellin A_5 to gibberellin A_3 in seedlings of dwarf *Pisum sativum*. Phytochemistry, 12: 1609-1612.

Eagles, C.F., 1967a. The effect of temperature on vegetative growth in climatic races of *Dactylis glomerata* in controlled environments. Ann. Bot. (n.s.) 31: 31-39.

Eagles, C.F., 1967b. Apparent photosynthesis and respiration in populations of *Lolium perenne* from contrasting climatic regions. Nature, 215: 100-101.

Eagles, C.F., and K.J. Treharne, 1969. Photosynthetic activity of *Dactylis glomerata* L. in different light regimes. Photosynthetica, 3: 29-38.

Eldridge, K.G., 1969. Altitudinal variation in *Eucalyptus regnans*. Ph.D. Thesis, Australian National University.

Eliasson, L., L.-H. Strömquist, and E. Tillberg, 1976. Reliability of the indolo-α-pyrone fluorescence method for indole-3-acetic acid determination in crude plant extracts. Physiologia Plantarum, 36: 16-19.

Elson, G.W., D.F. Jones, J.MacMillan, and P.J. Suter, 1964. Plant hormones. IV. Identification of the gibberellins of *Echinocystis macrocarpa* Greene by thin layer chromatography. Phytochemistry, 3: 93-101.

Engelbrecht, L., and K. Mothes, 1960. Kinetin als Faktor der Hitzresistenz. Ber. Dtsch. Bot. Ges., 73: 246-257.

Field, R.J., and D.I. Jackson, 1974. A hormone balance theory of apical dominance. pp. 655-657 in R.L. Bieleski, A.R. Ferguson and M.M. Cresswell (eds.), "Mechanisms of Regulation of Plant Growth". Bull. 12, Royal Society of New Zealand, Wellington.

Frankland, B., and P.F. Wareing, 1960. Effect of gibberellic acid on hypocotyl growth of lettuce seedlings. Nature, 185: 255-256.

Frydman, V.M., and J. MacMillan, 1973. Identification of gibberellins A_{20} and A_{29} in seed of *Pisum sativum* cv. Progress No. 9 by combined gas chromatography-mass spectrometry. Planta, 115: 11-15.

Galston, A.W., and M.E. Hand, 1949. Adenine as a growth factor for etiolated peas and its relation to the thermal inactivation of growth. Arch. Biochem. Biophys., 22: 434-443.

Glenn, J.L., C.C. Kuo, R.C. Durley, and R.P. Pharis, 1972. Use of insoluble polyvinylpyrrolidone for purification of plant extracts and chromatography of plant hormones. Phytochemistry, 11: 345-351.

Green, J.W., 1969. Temperature responses in altitudinal populations of *Eucalyptus pauciflora* Sieb. ex Spreng. New Phytol., 68: 399-410.

Gregory, F.G., and C.R. Hancock, 1955. The rate of transport of natural auxin in woody shoots. Ann. Bot. (n.s.), 19: 451-465.

Grunwald, C., J. Mendez, and B.B. Stowe, 1968. Substrates for the optimum gas chromatographic separation of indolic methyl esters and the resolution of components of methyl-3-indole pyruvate solutions. pp. 163-171 in F. Wightman and G. Setterfield (eds.), "Biochemistry and Physiology of Plant Growth Substances". The Runge Press, Ottawa.

Grunwald, C., M. Vendrell, and B.B. Stowe, 1967. Evaluation of gas and other chromatographic separations of indolic methyl esters. Anal. Biochem., 20: 484-494.

Hamilton, R.H., R.S. Bandurski, and B.H. Grigsby, 1961. Isolation of indole-3-acetic acid from corn kernels and etiolated corn seedlings. Plant Physiol., 36: 354-359.

Hartsema, A.M., 1961. Influence of temperatures on flower formation and flowering of bulbous and tuberous plants. Encycl. Plant Physiol., 16: 123-167.

Hertel, R., M.L. Evans, A.C. Leopold, and H.M. Sell, 1968. The specificity of the auxin transport system. Planta, 85: 238-249.

Hillman, W.S., and A.W. Galston, 1961. The effect of external factors on auxin content. Encycl. Plant Physiol., 14: 683-702. (Springer-Verlag, Berlin).

Honda, S.I., 1974. Fractionation of green tissue. Methods in Enzymology, 31: 544-553. (Academic, New York).

Hofstra, G., and J.D. Hesketh, 1969. The effect of temperature on stomatal aperture in different species. Can. J. Bot., 47: 1307-1310.

Ingersoll, R.B., and O.E. Smith, 1971. Transport of abscisic acid. Plant Cell Physiol., 12: 301-309.

Itai, C., and A. Benzioni, 1974. Regulation of plant response to high temperature. pp. 477-482 in R.L. Bieleski, A.R. Ferguson and M.M. Cresswell (eds.), "Mechanisms of Regulation of Plant Growth". Bull. 12, Royal Society of New Zealand, Wellington.

Jones, D.F., J. MacMillan, and M. Radley, 1963. Plant hormones. III. Identification of gibberellic acid in immature barley and immature grass. Phytochemistry, 2: 307-314.

Kapoor, M., M. O'Brien, and A. Braun, 1976. Modification of the regulatory properties of pyruvate kinase of *Neurospora* by growth at elevated temperatures. Can. J. Biochem., 54: 398-407.

Ketellapper, H.J., and J. Bonner, 1961. The chemical basis of temperature responses in plants. Plant Physiol., 36 (supp.): xxi.

Knegt, E., and J. Bruinsma, 1973. A rapid, sensitive and accurate determination of indolyl-3-acetic acid. Phytochemistry, 12: 753-756.

Kramer, P.J., 1957. Some effects of various combinations of day and night temperatures and photoperiod on the height growth of loblolly pine seedlings. Forest Sci., 3: 45-55.

Kramer, P.J., and T.T. Kozlowski, 1960. "Physiology of Trees". McGraw-Hill Book Company, New York, Toronto, London.

Lamontagne, N.S., and D.F. Johnson, 1970. Chromatography of adrenocortical steroids on silicic acid columns. J. Chromatog., 53: 225-232.

Langridge, J., 1963. Biochemical aspects of temperature response. Ann. Rev. Plant Physiol., 14: 441-462.

Langridge, J., and B. Griffing, 1959. A study of high temperature lesions in *Arabidopsis thaliana*. Aust. J. Biol. Sci., 12: 117-135.

Larcher, W., 1969. The effect of environmental and physiological variables on the CO_2 gas exchange of trees. Photosynthetica, 3: 167-198.

Leopold, A.C., and P.E. Kriedemann, 1975. "Plant Growth and Development". 2nd ed. 545 pp. Tata McGraw-Hill Publishing Company, New Delhi.

Levitt, J., 1969. Growth and survival of plants at extremes of temperature: a unified concept. Symp. Soc. Exp. Biol., 23: 395-448.

Levitt, J., 1972. "Responses of Plants to Environmental Stresses". 697 pp. Academic Press, New York and London.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall, 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-275.

MacMillan, J., and R.J. Pryce, 1968. Further investigations of gibberellins in *Phaseolus multiflorus* by combined gas chromatography-mass spectrometry. The occurrence of gibberellin A_{20} (*Pharbitis* gibberellin) and the structure of compound b. Tetrahedron Lett., 1537-1542.

MacMillan, J., and R.J. Pryce, 1973. The gibberellins. pp. 283-326 in L.P. Miller (ed.) "Phytochemistry". Vol. 3, 448 pp. Van Nostrand Reinhold Co., New York.

MacMillan, J., and P.J. Suter, 1963. Thin layer chromatography of the gibberellins. Nature, 197: 790.

MacMillan, J., R.J. Pryce, G. Eglinton, and A. McCormick, 1967. Identification of gibberellins in crude plant extracts by combined gas chromatography-mass spectrometry. Tetrahedron Lett., 2241-2243.

Mann, J.D., and E.G. Jaworski, 1970. Minimizing loss of IAA during purification of plant extracts. Planta, 92: 285-291.

McCrindle, R., and K.H. Overton, 1965. The chemistry of the cyclic diterpenoids. Advances in Organic Chemistry: Methods and Results, 5: 47-113.

McPherson, H.G., and R.O. Slatyer, 1973. Mechanisms regulating photosynthesis in *Pennisetum typhoides*. Aust. J. Biol. Sci., 26: 329-339.

Petinov, N.S., and U.G. Molotkovsky, 1957. Protective reactions in heat-resistant plants induced by high temperatures. Soviet Plant Physiol., 4: 221-228.

Pisek, A., W. Larcher, I. Pack, and R. Unterholzner, 1968. Kardinals temperaturbereiche der photosynthese und grenztemperaturen des lebens der blätter verschiedener spermatophyten. II Temperaturmaximum der netto-photosynthese und hitzresistenz der blätter. Flora, 158: 110-128.

Miller, C.O., 1951. Promoting effect of cobaltous and nickelous ions on expansion of etiolated bean leaf discs. Arch. Biochem. Biophys., 32: 216-218.

Miller, C.O., 1956. Similarity of some kinetin and red light effects. Plant Physiol., 31: 318-319.

Morse, R.N., and L.T. Evans, 1962. Design and development of CERES - an Australian phytotron. J. Agr. Eng. Res., 7: 128-140.

Mothes, K., and W. Baudisch, 1958. Untersuchungen über die Reversibilität der Ausbleichung grüner blätter. Flora, 146: 521-531.

Murofushi, N., N. Takahashi, T. Yokota, and S. Tamura, 1968. Gibberellins in immature seeds of *Pharbitis nil*. I. Isolation and structure of a novel gibberellin, GA_{20}. Agr. Biol. Chem., 32: 1239-1245.

Myers, R.M., 1940. Effect of growth substances on the abscission layer in leaves of *Coleus*. Bot. Gaz., 102: 323-338.

Paton, D.M., R.R. Willing, W. Nicholls, and L.D. Pryor, 1970. Rooting of stem cuttings of *Eucalyptus*: a rooting inhibitor in adult tissue. Aust. J. Bot., 18: 175-183.

Pisek, A., W. Larcher, A. Vegis, and K. Napp-Zinn, 1973. Plants : the normal temperature range. pp. 102-194 in H. Precht, J. Christopherson, H. Hensel and W. Larcher, "Temperature and Life". 779 pp. Springer-Verlag, Berlin.

Pitel, D.W., L.C. Vining, and G.P. Arsenault, 1971. Improved methods for preparing pure gibberellins from cultures of *Gibberella fujikuroi*. Isolation by adsorption or partition chromatography on silicic acid and by partition chromatography on Sephadex columns. Can. J. Biochem., 49: 185-193.

Powell, L.E., and K.J. Tautvydas, 1967. Chromatography of gibberellins on silica gel partition columns. Nature, 213: 292-293.

Pryce, R.J., and J. MacMillan, 1967. A new gibberellin (A_{17}) in the seed of *Phaseolus multiflorus*. Tetrahedron Lett., 4173-4175.

Pryce, R.J., J. MacMillan, and A. McCormick, 1967. The identification of bamboo gibberellin in *Phaseolus multiflorus* by combined gas chromatography-mass spectrometry. Tetrahedron Lett., 5009-5011.

Pryor, L.D., 1972. The selection of eucalypts for regeneration. Appita, 26: 35-38.

Pryor, L.J., and L.A.S. Johnson, 1971. "A Classification of the Eucalypts". 102 pp. Australian National University, Canberra.

Pryor, L.D., 1976. "The Biology of Eucalypts". Institute of Biology - Studies in Biology No. 61. Edward Arnold, London.

Pryor, L.D., and B. Clarke, 1964. Reforestation of former farm sites on the north coast of New South Wales. Aust. For., 28: 125-135.

Pryor, L.D., W.G. Chandler, and B. Clarke, 1968. The establishment of *Eucalyptus* plantations for pulpwood production in the Coffs Harbour region of New South Wales. A.P.M. Forests Pty. Ltd., Bull. No. 1.

Rosen, H., 1957. A modified ninhydrin colorimetric analysis for amino acids. Arch. Biochem. Biophys., 67: 10-15.

Scholander, P.F., H.T. Hammel, E.D. Bradstreet, and E.A. Hemmingsen, 1965. Sap pressure in vascular plants. Science, 148: 339-346.

Scurfield, G., 1961. The effects of temperature and day length on species of *Eucalyptus*. Aust. J. Bot., 9: 37-56.

Slatyer, R.O., 1977. Altitudinal variation in the photosynthetic characteristics of snow gum, *Eucalyptus pauciflora* Sieb. ex Spreng. III. Temperature response of material grown in contrasting thermal environments. Aust. J. Plant Physiol., 4: 301-312.

Slatyer, R.O., and P.J. Ferrar, 1977. Altitudinal variation in the photosynthetic characteristics of snow gum, *Eucalyptus pauciflora* Sieb. ex Spreng. II. Effects of growth temperature under controlled conditions. Aust. J. Plant Physiol., 4: 289-299.

Slatyer, R.O., and P.A. Morrow, 1977. Altitudinal variation in the photosynthetic characteristics of snow gum, *Eucalyptus pauciflora* Sieb. ex Spreng. I. Seasonal changes under field conditions in the Snowy Mountains area of south-eastern Australia. Aust. J. Bot., 25: 1-20.

Stetler, D.A., and W.M. Laetsch, 1965. Kinetin induced chloroplast maturation in cultures of tobacco tissue. Science, 149: 1387-1388.

Stoessl, A., and M.A. Venis, 1970. Determination of submicrogram levels of indole-3-acetic acid: a new, highly specific method. Anal. Biochem., 34: 344-351.

Takahashi, N., N. Murofushi, S. Tamura, N. Wasada, H. Hoshino, T. Tsuchiya, T. Aoyama, and H. Morita, 1967. High resolution mass spectra of gibberellins. Tetrahedron Lett., 895-899.

Takahashi, N., N. Murofushi, S. Tamura, N. Wasada, H. Hoshino, T. Tsuchiya, S. Sasaki, T. Aoyama, and E. Watanabe, 1969. Mass spectrometric studies on gibberellins. Org. Mass Spectrom., 2: 711-722.

Toprover, Y., and Z. Glinka, 1976. Calcium ions protect beet root cell membranes against thermally induced changes. Physiologia Plantarum, 37: 131-134.

Treharne, K.J., and C.F. Eagles, 1970. Effect of temperature on photosynthetic activity of climatic races of *Dactylis glomerata* L. Photosynthetica, 4: 107-117.

Ueda, M., and R.S. Bandurski, 1969. A quantitative estimation of alkali-labile indole-3-acetic acid compounds in dormant and germinating maize kernels. Plant Physiol., 44: 1175-1181.

Umbreit, W.W., R.H. Burris, and J.F. Stauffer, 1957. "Manometric Techniques". Burgess Publishing Company, Minneapolis.

Von Guttenberg, H., and H. Leike, 1958. Untersuchungen über den wüchs- und Hemmstoffgehalt ruhender und treibender Knospen von *Syringa vulgaris*. Planta, 52: 96-120.

Waldron, J.C., 1976. Nitrogen compounds transported in the xylem of sugar cane. Aust. J. Plant Physiol., 3: 415-419.

Wardlaw, I.F., 1972. Temperature and the translocation of photosynthate through the leaf of *Lolium temulentum*. Planta, 104: 18-34.

Wardlaw, I.F., 1974. Temperature control of translocation. pp. 533-538 in R.L. Bieleski, A.R. Ferguson, and M.M. Cresswell (eds.), "Mechanisms of Regulation of Plant Growth". Bull. 12, Royal Society of New Zealand, Wellington.

Went, F.W., and K.V. Thimann, 1937. "Phytohormones". 294 pp. MacMillan, New York.

West, C., G.E. Briggs, and F. Kidd, 1920. Methods and significant relations in the quantitative analysis of plant growth. New Phytol., 19: 200-207.

Wetmore, R.H., and W.P. Jacobs, 1953. Studies on abscission: the inhibiting effect of auxin. Am. J. Bot., 40: 272-276.

Whitehead, F.H., and P.J. Myerscough, 1962. Growth analysis of plants. The ratio of mean relative growth rate to mean relative rate of leaf area increase. New Phytol., 61: 314-321.

Williams, R.F., 1946. The physiology of plant growth with special reference to the concept of net assimilation rate. Ann. Bot. (n.s.), 10: 41-72.

Wilson, B.F., and E.P. Bachelard, 1975. Effects of girdling and defoliation on root activity and survival of *Eucalyptus regnans* and *E. viminalis* seedlings. Aust. J. Plant Physiol., 2: 197-206.

Yamaguchi, I., T. Yokota, N. Murofushi, Y. Ogawa, and N. Takahashi, 1970. Isolation and structure of a new gibberellin from immature seeds of *Prunus persica*. Agr. Biol. Chem., 34: 1439-1441.